Hepatic Injury in Metabolic Syndrome: The role of selenium in models of hepatic injury and healing

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MBChB

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Dedication

This thesis is dedicated to:

My great parents, Hussein and Sanaa, who raised me, taught me and supported me. They have been with me every step of the way, through good and bad times. Thank you for all the unconditional love, guidance and support that you have always given me, helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. Thank you for everything. I love you!

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Declaration

I, Hussam Baghdadi, declare that the following dissertation is entirely my own work. This work is original and the results presented in this thesis are my own work except the following:

a. The Oil red-O staining of lipid in the C3A cells was kindly done by Dr Forbes Howie.
b. The cytokine assessments were kindly done by Dr. Susan Mackenzie.
c. The blood samples were kindly collected by Professor Peter Hayes, Hepatology Unit, Royal Infirmary of Edinburgh.
d. The selenium measurements of blood samples were kindly done by Mr Gordon Marr.

No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Hussam Baghdadi

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ANOVA	Analysis of variance
BMI	Body mass index
BSA	Bovine albumin serum
Cpm	Count per minute
CV	Coefficient of variation
Cys	Cysteine residue
DMEM	Dulbecco's modified Eagle's medium
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Glutathione disulphide (oxidised glutathione)
GST	Glutathione-S-transferase
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
4-HNE	4-hydroxy-2-nonenal
H_2O	Water
H_2O_2	Hydrogen peroxide
hr	Hour
HSC	Hepatic stellate cells
IL-8	Interleukin-8
kDa	Kilodalton
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LFT	Liver function test
LOX	Lysyl oxidase

MBq	Mega Becquerel
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFL	Non-alcoholic fatty liver
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFκB	Nuclear factor κB
NO	Nitric oxide
Pd/Mg	Palladium/magnesium
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PHGPX	Phospholipid hydroperoxide glutathione peroxidase
PPAR	Peroxisomal proliferator-activated receptor
PPC	Polyenylphosphatidylcholine
qPCR	Quantitative PCR
ROS	Reactive oxygen species
SD	Standard deviation
Se	Selenium
sec	Seconds
SECIS	Selenocysteine insertion sequence
SeCys	Selenocysteine
STS	Sulphatase transferase
t-BuOOH	Tert-butylhydroperoxide
TG	Triglyceride
TGF-β1	Transforming growth factor-β1
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-α
TR	Thioredoxin reductase
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
Trx	Thioredoxin

Abstract

Oxidative stress, lipid peroxidation, and endotoxaemia with cytokine-mediated injury have been implicated as factors in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). The degree of insulin resistance together with co-existing inadequacies of vital antioxidant defence mechanisms may be important determinants of progression to fibrosis in patients with non-alcoholic steatohepatitis (NASH). Current therapies are targeted at improving insulin sensitivity as well as addressing hepatic repair including anti-inflammatory strategies. Anti-oxidants remedies have also been tested but the role of selenoenzymes with antioxidant action, namely thioredoxin reductase 1 (TR1) and glutathione peroxidase 1 (GPX1) have been ignored.

The aim of this thesis is to investigate the role of selenium in the pathophysiology of NAFLD both *in vitro* and *in vivo*. The *in vitro* studies used cell lines representing the cell types involved in the disorder; hepatocytes (C3A line) and hepatic stellate cells (LX-2 line). In order to assess the influence of selenium status and selenoenzymes expression on the pathogenesis of NAFLD it was necessary to develop a culture system which allowed good cell viability in selenium free culture medium. This was achieved by the use of an insulin and transferrin (IT)-supplemented medium which importantly was free of any animal serum additions. Using this IT culture medium, selenium addition (as selenite) produced a significant increase in the expression of GPX1 and TR1 in both C3A and LX2 cells. TR1 and GPX1 were expressed at similar levels in both C3A and LX-2 cells.

It was also necessary to develop an in-vitro model for fat loading C3A cells to mimic fatty liver pathophysiology. Two models of fat loading were investigated. One model used lactate, pyruvate, octanoate and ammonium (LPON). LPON has been previously used to increase the functionality of C3A cells but it was observed that fat droplets accumulated in these LPON treated cells. Dissection of the agents in the LPON revealed that octanoate was the factor that increased the triglyceride

accumulation. Interestingly, octanoate also increased the expression of TR1 and GPX1, suggesting that it could induce oxidative stress leading to the induction of selenoenzymes to afford a protective defence mechanism. In the second model, oleate and/or palmitate were used to fat-load C3A cells. These cells had significantly higher triglyceride content than the LPON-fat-loaded cells. However, oleate and/or palmitate treatments did not increase the expression of either TR1 or GPX1 in C3A cells suggesting perhaps these cells were not under oxidative stress. LPON and oleate/palmitate were also capable of fat loading LX2 cells.

Selenium-supplementation of C3A and LX-2 cells efficiently protected (measured by their lactate dehydrogenase retention) them from oxidative damage induced by t-butylhydroperoxide. This suggests that selenium supplementation through its incorporation into selenoenzymes could protect the cells from the oxidative damage.

The role of selenium was also investigated in the regulation of α-1 pro-collagen mRNA expression. In LX-2 cells, the expression of α-1 pro-collagen mRNA was unaffected by the selenium status of the cell. Similarly the selenium status of C3A cells had no effect on modifying α-1 pro-collagen mRNA of LX2 cells when co-culture or conditioned medium experiments were performed. These results suggest that LX-2 cells were already largely activated and at a stage unable to be ameliorated by selenium treatment. In contrast, studies on C3A cells revealed that TGF-β1 (common inducer of α-1 pro-collagen mRNA in hepatic stellate cells) dramatically increased the expression of α-1 pro-collagen mRNA in C3A cells to the levels observed in LX-2 cells. More interestingly, selenium supplementation of C3A cells notably decreased α-1 pro-collagen mRNA expression in response to TGF-β1.

In the *in vivo* study, plasma selenium in type 2 diabetics (high risk of developing NAFLD) were inversely related to the body mass index and in most patients selenium levels were below that required to maximally express GPX1 in red cells. Furthermore, type 2 diabetics had lower plasma selenium levels compared to the healthy control group. Collectively, this suggests that in the UK population, obesity

is a risk factor for both insulin resistance and decreased selenium status leading to sub-optimal antioxidant protection.

In conclusion, this study provides evidence that selenium through increasing the expression of selenoenzymes is beneficial in protecting liver cells from oxidative stress. Furthermore, selenium is capable of suppressing α-1 pro-collagen mRNA expression in hepatocytes although not in activated hepatic stellate cells. Taken together these data support the view that suboptimal selenium intake in the UK may be a risk factor in the pathogenesis of NAFLD.

1 CHAPTER ONE

INTRODUCTION

1.1 Non-alcoholic fatty liver disease (NAFLD)

Patients with hepatic fibrosis are usually clinically asymptomatic but may have in their plasma modest elevations of liver enzymes such as ALT, AST and GGT. Histologically, hepatic fibrosis is characterized by an increase in extracellular matrix constituents which are produced in response to chronic liver injury (Friedman, 2000). However, collagen production by the liver is also an essential physiological response to tissue injury that is believed to be part of the wound healing and tissue recovery process. It is thought that a persistent injury may provoke inflammatory processes which collectively form the hepatic scar found in chronic liver disease (Bataller and Brenner, 2005). Alcoholic fatty liver (AFLD), metabolic derangements (NAFLD), persistent viral and helminthic infections, and hereditary metal overload are some of the known causes of liver fibrosis (Lotersztajn et al., 2005). In many western societies, NAFLD is now thought to be the most common cause of elevated liver enzymes (Day, 2002).

The fatty change of the liver observed in NAFLD has different stages. It starts with accumulation of triglyceride but without any noticeable inflammation (NAFL). In some patients this quiescent state can progress to produce inflammation and become Non-Alcoholic SteatoHepatitis (NASH) and at this stage abnormalities in the serum transaminases and gamma-glutamyl transferase (GGT) may become apparent. If this inflammation persists and becomes chronic, approximately 20% of NASH cases may progress to fibrosis and ultimately cirrhosis over a 20-30 year period (Malnick et al., 2003) (see figure 1).

Historically, it was only in 1980 that the term ‘non-alcoholic steatohepatitis’ (NASH) was first introduced. The term described a condition that appeared similar to

alcoholic liver disease but in which alcohol played no part in the pathogenesis. It was also realised that the condition was often associated with metabolic syndrome (Younossi, 1999).

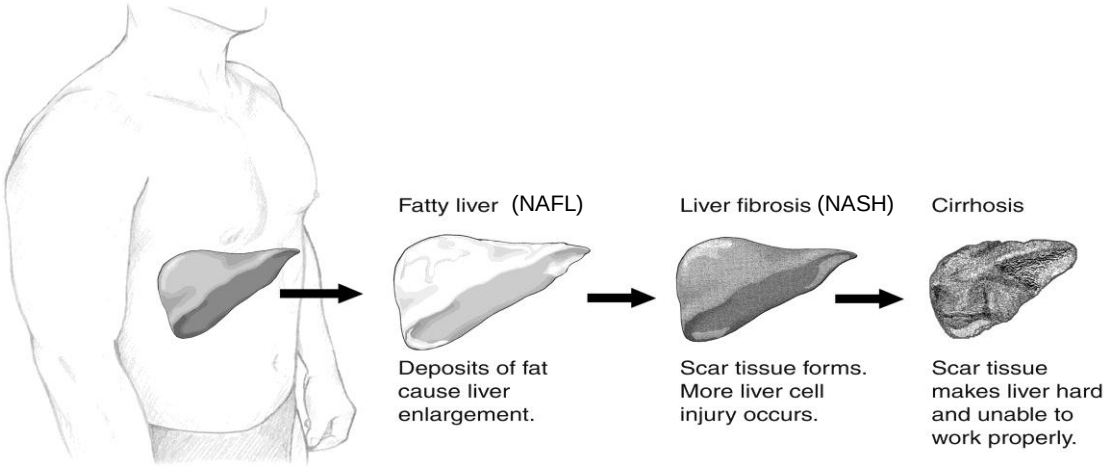


Figure 1.1: NAFLD stages of disease progression: The disease starts with mild fat accumulation in the liver which might present as a liver enlargement but in most of the cases, it is asymptomatic. Development of inflammation may then occur and the formation of a pathological scar is apparent. Persistent NASH can then lead to cirrhosis. Figure adapted from National Digestive Diseases Information Clearinghouse web site (http://digestive.niddk.nih.gov/spanish/pubs/nash/).

Recognition, staging and treatment of NAFL and NASH are important to prevent or delay their progression into liver cirrhosis or carcinoma. Current therapies usually relate to improvement of insulin sensitivity including gradual weight loss, physical exercise and drugs to increase insulin sensitivity as well as the use of agents that address hepatic repair including anti-inflammatory and anti-oxidant strategies.

1.1.1 Causes, epidemiology, clinical features, investigations, complications and clinical management.

Causes

NAFLD can be either a primary or a secondary disease. The primary form of NAFLD is usually associated with the metabolic syndrome. Secondary NAFLD could arise from different causes such as starvation, or taking certain medications, including glucocorticoids, tamoxifen and amiodarone (Ong and Younossi, 2007). Table 1.1 list the secondary causes of NAFLD.

Table 1.1: The secondary causes of NAFLD.

Secondary causes of NAFLD	
Nutritional	Total parental nutrition, rapid weight loss, starvation, intestinal bypass surgery
Drugs	Glucocorticoids, estrogens, tamoxifen, methotrexate, zidovudine Amiodarone, ASA, intravenous tetracycline, didanosine, cocaine, perhexilene, hypervitaminosis A, diltiazem
Toxins	Toxic mushrooms (Amanita phalloides, Lepiota) Petrochemicals, phosphorus, Bacillus cereus toxin
Metabolic	Lipodystrophy, dysbetalipoproteinemia, Weber–Christian disease, Wolman's disease Acute fatty liver of pregnancy, Reye's syndrome Other inflammatory bowel disease, HIV infection, small-bowel diverticulosis with bacterial overgrowth

Adapted from (Adams et al., 2005)

Epidemiology

With the emerging epidemic of obesity in western countries, associated hepatic steatosis has been found in approximately 20-30% of the population in US when assessed by the magnetic resonance spectroscopy (Adams et al., 2005; Kim and Younossi, 2008). Other studies reported that fatty liver could be detected in approximately 10-24% (Medina et al., 2004). Evidence suggests that the prevalence of fatty liver increases with age such that it is about 10 times more common in patients aged 40 – 60 years of age than in children (Adams et al., 2005). However, the prevalence of NAFLD is more frequent in diabetics (50%) and the obese (76%) and can be found in almost all patients who are morbidly obese (Adams et al., 2005). Other factors found to be associated with the prevalence of NAFLD include hyperlipidemia (hypertriglyceridemia, hypercholesterolemia, or both) which has been observed in 20% to 81% of patients with NASH (Sheth et al., 1997). Although commonly NAFLD is associated with obesity or diabetes, NAFLD can develop in patients with normal body weight, normal blood sugar and normal lipid profiles (Sheth et al., 1997).

Clinical features

Most of the patients with NAFLD present to clinics because of other associated clinical problems including obesity, diabetes or hypertriglyceridemia. Only a minority of patients complain of features of liver disease such as non-specific right upper abdominal pain and fatigue (Sheth et al., 1997). On examination, such patients may have enlargement of the liver but more often than not no clinical signs of chronic liver disease are detected (Adams et al., 2005).

Investigations

In a case of suspected NAFLD, investigations would be requested to exclude other potential diagnoses. Most NAFLD patients are characterized by minor but persistent abnormalities in liver function tests but it remains important to exclude other pathologies that could give rise to these abnormal tests (Sheth et al., 1997).

The diagnosis of NAFLD is done by exclusion of other differential diagnoses including alcoholic liver disease, hepatitis C, B and other causes of fatty liver. The commonly used cut-off limit of alcohol intake per day to distinguish AFLD from NAFLD is 20g/day for females and 30g/day for males (Adams et al., 2005). Although, elevated liver enzymes could be an indication of NAFLD, especially an ALT:AST ratio > 1 in early disease, clinicians have suggested that the diagnosis of NAFLD relies on two parameters:

- Exclusion of other liver diseases by conventional tests
- At least moderate macrovesicular fatty change associated with inflammation on liver biopsy (Sheth et al., 1997).

If bilirubin and alkaline phosphatase levels are slightly elevated, this might indicate a later stage of the disease (Patrick, 2002). If the RBC mean corpuscular volume (MCV) is raised, this might indicate alcoholic liver disease (Maruyama et al., 2001). The level of abnormality of liver function tests does not correlate well to the histological changes in the liver in NAFLD (Adams et al., 2005). Ferritin and autoantibodies may be elevated but are not recognized as a diagnostic feature (Adams et al., 2005). As a consequence it is often difficult from a clinical point of view to determine the severity of liver dysfunction and fibrosis in NAFLD without resort to liver biopsy.

Other laboratory tests used to exclude other causes of liver disease include serologic tests for viral hepatitis, iron studies for haemochromatosis, ceruloplasmin levels for Wilson's disease and anti-mitochondrial and antinuclear antibodies for autoimmune hepatitis (Adams et al., 2005).

Liver ultrasonography and CT shows diffuse fatty infiltration in patients with NAFLD (Adams et al., 2005). However, this finding is not specific to NAFLD and cannot be used to diagnose NASH. Since the BMI of the typical NAFLD patient is high, the sensitivity and the specificity of ultrasonography ability to detect any fatty infiltration are often compromised (Adams et al., 2005). The liver biopsy is usually

done to either confirm or exclude NAFLD if none of the above tests are conclusive(Adams et al., 2005; Sheth et al., 1997).

Liver biopsy has been proposed as a diagnostic tool to determine the severity and the stage of the disease (Adams et al., 2005). The decision to take a liver biopsy should be discussed with the patient and at the same time the benefits and risks of the biopsy should be explained (Adams et al., 2005). Under the microscope, the histological changes may not always be able to distinguish alcoholic liver disease from NAFLD. Generally, there is a mix of macrovesicular and microvesicular droplets along with defective mitochondria (Adams et al., 2005; Sheth et al., 1997).

Complications

Cirrhosis and hepatocellular carcinoma are the main complications of NAFLD and more specifically NASH patients. Both of these complications lead to end stage liver failure, renal failure, portal hypertension, variceal haemorrhage, ascites, and encephalopathy. Once developed, these hepatic complications may recur after liver transplantation (Adams et al., 2005).

Clinical management

Weight reduction, lowering of serum lipids, exercise and medication to improve insulin resistance (metformin and thiazolidinediones are commonly tried) are currently the broad lines of treating NAFLD patients. The main aim of the treatment is to slow, or more preferably, prevent the disease progression by managing the associated clinical problems (Adams et al., 2005). Rapid weight loss should be avoided and the ideal weight loss should not exceed 10% of the body weight in 6 months (Adams et al., 2005). Liver transplantation is the treatment reserved for complicated cases of cirrhosis (Adams et al., 2005).

Table 1.2: Histological feature of NASH

Histopathology of NASH	
• Necessary components	
	- Steatosis (macro > micro) - Hepatocellular ballooning - Mixed mild lobular inflammation
• Usually present	
	- Zone 3 perisinusoidal/pericellular fibrosis - Zone 1 hepatocellular glycogenated nuclei - Small lipogranulomas, acidophil bodies, PAS-positive Kupffer cells
• Maybe present	
	- Mallory's hyaline - Hepatocellular iron granules in zone 1 - Megamitochondria
• Unusual for NASH	
	- Predominantly microvesicular steatosis - Sclerosing hyaline necrosis - Veno-occlusive lesions, phlebosclerosis, perivenular fibrosis - Portal changes - Acute/chronic cholestasis

Adapted from (Jansen, 2004)

1.1.2 Pathogenesis of NAFLD

Generally, the pathogenesis of NAFLD can be regarded as a chronic wound healing process with or without inflammation, characterized by increased deposition of extra-cellular matrix (Bataller and Brenner, 2005; Friedman, 2000). At the cellular level, most of the literature describes the pathogenesis at NAFLD based on a two-hit hypothesis. The first hit is insulin resistance while the second hit is unopposed oxidative stress (Day and James, 1998). Both 'hits' will be described in the following section.

1.1.2.1 Two-hit hypothesis of fatty liver

1.1.2.1.1 Insulin resistance

The general physiological action of insulin is principally anabolic, i.e., to build energy stores and body mass. Insulin facilitates the transportation and entry of glucose to the cells of the various tissues. In the liver, insulin induces the synthesis of glycogen from glucose. Collectively, insulin targets the reduction of the blood sugar level, especially after heavy meals. Insulin also promotes the synthesis of fatty acids in the liver and inhibits the oxidation of fatty acids in the liver and adipose tissue.

Insulin resistance describes a state where tissues become unresponsive to insulin. Insulin resistance leads to continuous secretion of insulin (hyperinsulinemia) from the β-cells of the pancreas to overcome the tissue resistance. Furthermore, insulin resistance increases the lipolysis of fatty acids from the adipose tissue leading to a huge influx of fatty acids from the adipose tissue to the liver. Insulin will also stimulate the lipogenic pathway in the liver through the sterol regulatory element-binding protein1 (SREBP-1) (Browning and Horton, 2004; Leclercq et al., 2007). Moreover, insulin resistance will lead to a decrease in lipoprotein synthesis and a consequent reduction in the export of fat from the liver in the form of very-low-density lipoprotein (VLDL) (Jansen, 2004). Accumulated fatty acids in the hepatocyte cytoplasm can activate the peroxisomal proliferator-activated receptor-α

(PPARα) which stimulates the peroxisomal and mitochondrial oxidation of fatty acid and therefore production of reactive oxygen species (ROS) (Jansen, 2004) (see figure 1.2). These mechanisms ultimately will lead to the accumulation of fatty acids in the liver that may be oxidised or stored as triglycerides (fat)

Peripheral versus hepatic insulin resistance

Peripheral insulin resistance usually refers to resistance in tissues (i.e. adipose and muscle) other than liver. On the other hand, hepatic insulin resistance refers to the unresponsiveness of hepatocytes to insulin action and all the consequences resulted from this deranged unresponsiveness.

However, peripheral insulin resistance can lead to inhibition of hormone-sensitive lipase and ultimately to lipolysis of fatty acids from adipose tissue. Furthermore, peripheral insulin resistance can lead to increased *de novo* synthesis of fat in the liver and ultimately hepatic insulin resistance (Kim et al., 2001). The activation of protein kinase C (PKC) and c-Jun N-terminal protein kinase 1 (JNK1) that affect insulin signalling could be the mechanism by which the fat accumulation leads to hepatic insulin resistance (Samuel et al., 2004). Furthermore, other reports suggest that an activated protein kinase C (PKC theta) could induce serine phosphorylation of insulin receptor substrate (IRS-1) in muscle and IRS-2 in liver which in turn leads to defects in insulin actions in these tissues (Kim et al., 2001).

However, the continuous synthesis and accumulation of fatty acids in the liver can worsen the peripheral insulin sensitivity by blocking insulin action and increasing the risk of oxidative stress by providing its substrate (fatty acids) (McAvoy et al., 2006). This view is supported by a study suggesting that hepatic insulin resistance worsens with the progression from NAFL to NASH (Sanyal et al., 2001). Moreover, improving insulin action through changing lifestyle and pharmacological treatment decreases the steatosis. This suggests that insulin resistance predisposes the liver to accumulate fat and not vice versa. This was supported by the demonstration that

hepatic hyperinsulinaemia is not the cause of insulin resistance (Pagano et al., 2002). Therefore, it seems possible that fat accumulation and insulin resistance are intimately linked having the ability to promote each other.

Insulin resistance and inflammation

It has been argued the continuous mitochondrial oxidation of fatty acids in a fatty liver can lead to the production of a range of oxidation by products such as ROS and lipid hydroperoxides (Jansen, 2004). Ultimately, these lead to mitochondrial dysfunction and mitochondrial damage that in turn can lead to release of inflammatory cytokines thereby attracting inflammatory cells such as neutrophils and Kupffer cells (Parkes and Templeton, 2003; Reeves and Friedman, 2002). Activated Kupffer cells will then in turn release various types of cytokines to recruit other inflammatory cells and thus initiating an inflammatory cascade (Reeves and Friedman, 2002). This hypothesis is supported by a study that showed fat-loaded hepatocytes had increased ROS production (Nardo et al., 2001).

Various pro-inflammatory cytokines have been implicated in the process of liver fibrosis including TGF-β1, PDGF, TNF-α, IL-8 and leptin. TNF-α can induce insulin resistance and inflammation (Adams et al., 2005). Leptin, like adiponectin, is also released from the adipose tissue. Leptin has been described to be involved in regulating insulin resistance with regards to adiposity. Although genetically modified leptin-deficient mice develop less fibrotic change in their liver compared to wild type mice, the exact pathological role of this adipokine is not fully understood (Diehl et al., 2005). It has been suggested that as the fat-loaded hepatocytes become bigger microvascular blood flow may become impaired and thus may prevent the removal of the ROS and thereby exacerbating inflammation and fibrosis (Adams et al., 2005).

During inflammation, significant numbers of leucocytes are recruited. Neutrophils, Kupffer cells, monocytes and T-lymphocytes are among the most pro-inflammatory of cells that migrate to the inflamed liver (Kershenobich Stal and Weissbrod, 2003). These attracted immune cells contribute further to the inflammatory process and

through the release of various inflammatory cytokines which may activate hepatic stellate cells (Kershenobich Stal and Weissbrod, 2003). The exact role of each immune cell in the processes of stellate cell activation and fibrogenesis remains uncertain.

The duration and the severity of inflammation might be one of the important determining factors responsible for switching the normal wound-healing process in the liver to a fibrogenic process. The most important determinants of the factors involved in the pathogenesis of fatty liver are thought to be genetic polymorphisms in a range of genes including those which exhibit anti-oxidant and anti-inflammatory function. These genetic variations may determine the susceptibility of patients to be more prone to liver damage caused by fatty change and oxidative stress (McAvoy et al., 2006).

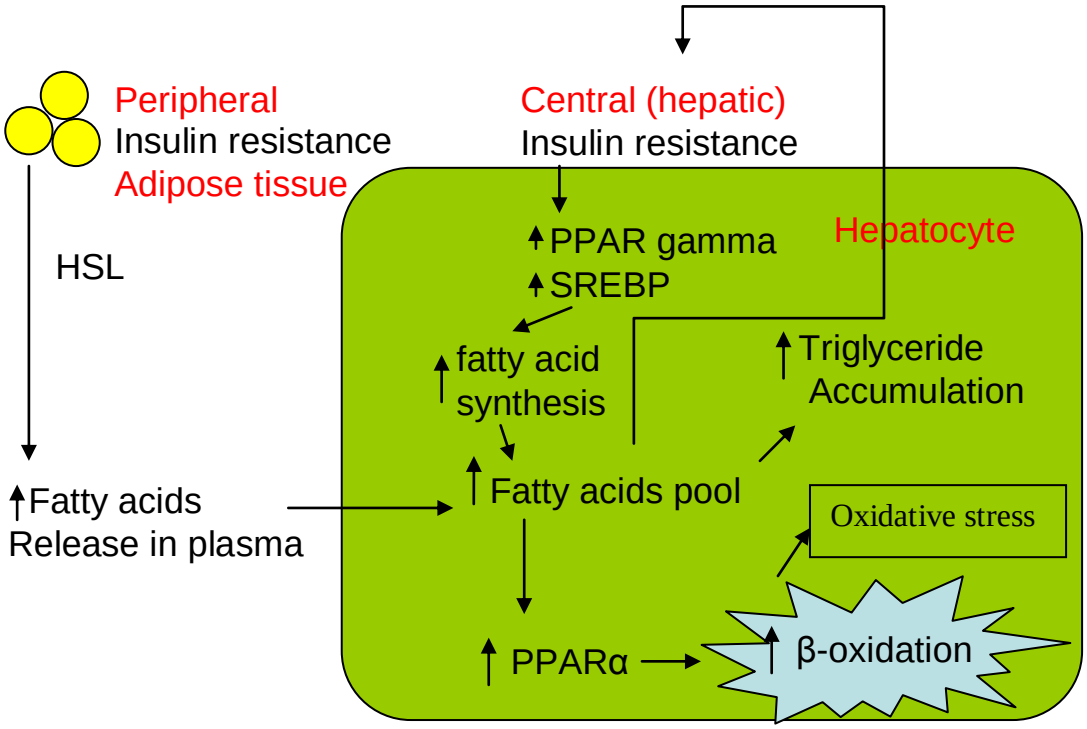


Figure 1.2: The main pathophysiological mechanism of NAFLD
HSL:Hormone sensitive lipase, PPAR: Peroxisome proliferator-activated receptor, SREBP: sterol regulatory element-binding protein. Adapted from (Fromenty et al., 2004).

1.1.2.1.2 Oxidative stress

Oxidative stress describes the process of accumulating reactive oxygen species (ROS) caused by either their over-production or failure of the anti-oxidant defence systems to prevent their accumulation (Sies, 1997). Oxidative stress plays a major role in many diseases including cancer, ageing, cardiovascular diseases, diabetes mellitus and alcoholic liver disease (Valko et al., 2007). Many regard unopposed oxidative stress as being the second hit of the hypothesis for the pathogenesis of NAFLD described earlier in this chapter. Although ROS are primarily toxic and harmful to cells, they also have to a limited extent a physiological role. One physiological role of ROS is to kill bacteria phagocyted by monocytes and other leucocytes involved in immunity (Valko et al., 2007). ROS are also involved in various signal transduction pathways (Valko et al., 2007).

Harmful effects of ROS vary from cell death through necrosis and apoptosis to less severe effects including modification of cell functionality and various metabolic processes (Valko et al., 2007). ROS can be classified according to their half lives. ROS with short lives have a more destructive potential than those with long half lives but are only capable of exerting a local effect within the cell where they are generated. In contrast ROS with a long half life have less destructive potential but at the same time can travel to other cells to produce oxidative damage (Sies, 1997). ROS interact with receptors which in turn may affect the binding or signal transduction pathways in the cell (Valko et al., 2007). In addition ROS can react with fatty acids and lipoproteins to generate harmful peroxyl radicals, aldehydes (e.g., 4-hydroxynonenal) and other products of lipid peroxidation. These lipid peroxidation products may also affect receptor function. 4-hydroxynonenal is a strong chemo-attractant for neutrophils (Blasig et al., 1995).

The biochemical consequences of oxidative stress vary. They include not only DNA oxidation and mitochondria necrosis but many others (see table1.3) (Parola and Robino, 2001). Although ROS can arise from various cell organelles, mitochondria are regarded as a very important site for their production since the high metabolic activity of the organelle leads to large amounts of oxygen being used (Parola and

Robino, 2001). Oxidative phosphorylation in the mitochondria to produce ATP from fat and pyruvate is a complex process by which most of the ROS is produced as a by-product. This process mainly involves the transfer of electrons from donor proteins to acceptor proteins. This mechanism of electron transfer can be associated with a leak of electrons and uncoupling of the oxidative mechanism. Recently, a study found that most of the ROS produced in the mitochondria is at the level of complex III because this complex might send ROS to the inter-membrane space where there are no antioxidant defence mechanisms (Chen et al., 2003). Therefore in stressed states where the anti-oxidant systems are exhausted, large amounts of unopposed ROS could be very harmful and damage mitochondria. Furthermore, the loss of glutathione stores has been found to be lethal in ascorbatic rats (Meister, 1995).

Table 1.3: The damaging effect of major oxidative stress-related molecules on different macromolecule

ROS (Reactive Oxygen species)	DNA: oxidation, strand breaks, genotoxicity Proteins: oxidation, fragmentation, formation of carbonyls Lipids: lipid peroxidation and degradation
HAK (Hydroxy Alkenals)	DNA: adducts (low doses), strand breaks, genotoxicity (high doses) Proteins: adducts (Michael type reactions on Lys, Cys and His residues)
NO (Nitric Oxide)	DNA: oxidation, strand breaks Proteins: oxidation, nitrosation, nitration (nytrosylation of tyrosine) Lipids: lipid peroxidation and degradation

Adapted from (Parola and Robino, 2001).

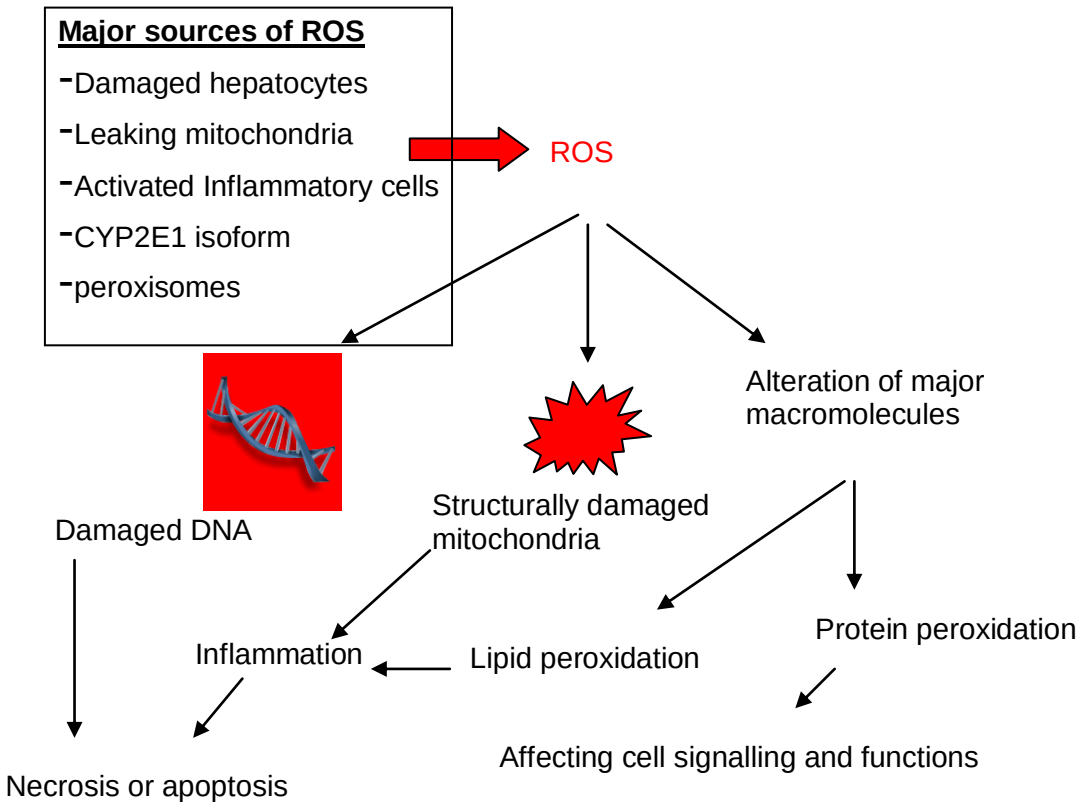


Figure 1.3: Oxidative stress related molecules sources and consequences
Adapted from (Parola and Robino, 2001).

Hepatic stellate cells and oxidative stress

Hepatic stellate cells (HSC) constitute about 15 % of the total cell population in the liver (Friedman, 2008). These cells were first described by von Kupffer in 1876 as liver sternzellen (star-shaped cells) (Friedman, 2008; Wake, 2006). In the quiescent state, HSCs are perisinusoidal cells that reside in the space of Disse and contain numerous droplets that are packed full with retinoids (Wake, 2006). The known basic functions of stellate cells are to store retinoids and regulate sinusoidal blood flow (Friedman, 2008). In liver fibrosis, stellate cells become activated and are regarded as the most important source of the extracellular matrix (Friedman, 2008). For this reason, the mechanisms by which hepatic stellate cells become activated have been studied widely.

The activation of stellate cells has two stages, an initiation and a perpetuation stage (Friedman, 2000). In the initiation phase, various factors have been implicated in the activation process including ROS, inflammatory cytokines and growth factors released from injured hepatocytes, Kupffer cells, and sinusoidal endothelial cells (see figure 1.4) (Friedman, 2000). Furthermore, the stellate cells express several transcription factors which may eventually lead to the development of a phenotypic transformation to activated myofibroblast-like cells. These transformed cells then express various cytoskeletal markers such as α-smooth muscle actin (α-SMA) and glial fibrillary acidic protein (GFAP) (Friedman, 2008). The activated stellate cells become enlarged, develop increased rough endoplasmic reticulum and lose their vitamin A (retinoid) droplets. Whilst in vitro studies have shown that activation of stellate cells is accompanied by loss of these vitamin A-rich droplets, to date there is no clear evidence to support a direct role for these lipid droplets in cell activation *in vivo* (Friedman et al., 1993). The hepatic content of vitamin A is decreased in liver fibrosis but again there is no evidence to show a convincing role of vitamin A in stellate cell activation (Friedman, 1993). Thus, the role of retinoids in the activation process remains unclear.

In the perpetuation phase, stellate cells become more active, proliferative and contractile (Friedman, 1993). Autocrine and paracrine stimuli together with the activated phenotype constitute the main factors involved in the perpetuation phase. Although various cytokines are involved in the process of activation, TGF-β1 is considered the main cytokine involved in the activation process and thus fibrogenesis. Furthermore, platelet-derived growth factor (PDGF) is regarded as the most potent inducer of stellate cell proliferation (Friedman, 1993).

The severity of liver fibrosis depends on the type and duration of the liver injury. In the case of acute injury, the regeneration process takes over the fibrogenesis process. On the other hand, if the injury is chronic, the regeneration process will be suppressed in the face of an ongoing fibrogenesis process (Friedman, 1993).

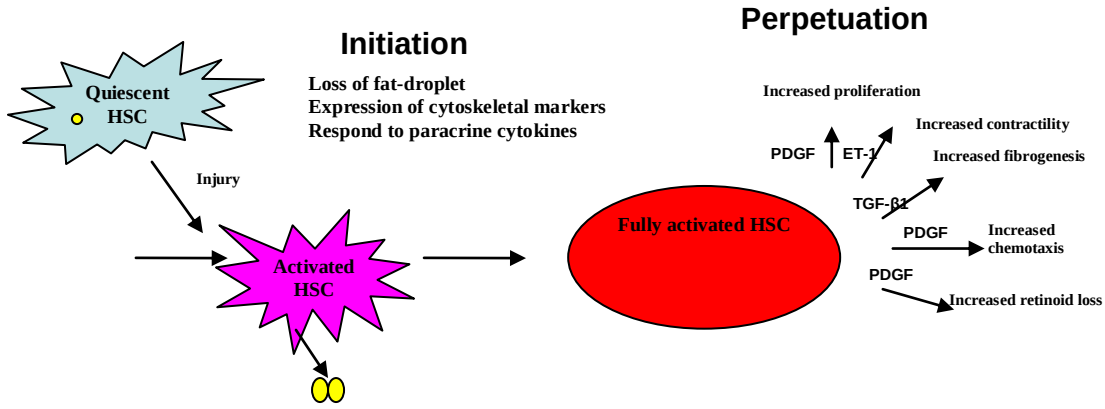


Figure 1.4: Stages of hepatic stellate cell activation: yellow droplets represents fat droplets in hepatic stellate cell (HSC).

1.1.3 The role of selenium and selenoenzymes in NAFLD

Selenium (Se) is a dietary trace mineral that has powerful anti-cancer, antioxidant and anti-inflammatory properties. It is also able to modify immune and endocrine function in humans and animals.

1.1.3.1 Selenium

Selenium is a trace element that is essential for a number of cellular functions. Selenium (selen in Greek means 'moon') was discovered by Berzelius in 1817. Although initially it was the toxic properties of selenium that generated scientific interest, Schwartz and Foltz in 1957 found that selenium could prevent hepatic necrosis in vitamin E-deficient rats (Schwarz and Foltz, 1957). This observation led to the acceptance that selenium was an essential trace element. In 1973, selenium was shown to be a constitutive part of cytoplasmic glutathione peroxidase (GPX1), an important enzymatic antioxidant. This discovery provided a plausible mechanism by which the trace element could exert many of its biological actions (McKenzie et al., 2002). Subsequently it has been found that selenium is a constituent part of a large number of selenoproteins which have functions other than an antioxidant role. The trace element, acting independently of selenoproteins, may also have biological actions including its ability to modify a range of cell signalling pathways including that of insulin (Beckett and Arthur, 2005) .

Selenium and the diet

Selenium exists in nature as both inorganic and organic forms. Inorganic forms include selenides, selenites and selenates whilst organic forms such as selenomethionine and selenocysteine are incorporated into proteins. Selenium enters the food chain by plants trapping inorganic forms of selenium from the soil and incorporating them into plant protein as selenomethionine and selenocyteine. There is wide variation in the selenium content of soil across the globe and in areas where

the selenium content of soil is low, populations relying on local food supplies become selenium deficient to varying degrees.

The dose of selenium that allows the maximal expression of glutathione peroxidases in red blood cells is currently considered as the recommended daily allowance of selenium. For humans, this is in the order of ~70 µg/day (Rayman, 2000). The selenium intake in the UK had fallen from about 60 µg/day during the 1970s to currently an average of about 30 µg/day nowadays (Combs, 2001). This decrease in selenium intake in the UK was mostly due to a change to the sourcing of wheat from European counties (which have low selenium soils) instead of areas of North America which have high selenium content in the soil. It continues to be debated if this current low intake of selenium in the UK diet has adverse health consequences.

Selenium is usually taken in the diet as organic selenium forms mainly from cereal products. However, inorganic selenium forms are commonly used in experimental *in-vitro* settings because of their speed of action. Selenium can be incorporated into both selenoproteins that are metabolically active and selenium-containing proteins that have no known biological role. Selenium is usually absorbed in the duodenum (Thomson, 1998). Although the various forms of selenium have different mechanisms of absorption, the rate of absorption is not regulated by the selenium level. While organic forms are actively absorbed using amino acid transporters (McConnell and Cho, 1965), inorganic forms can pass passively across the intestine (Thomson, 1998). Once selenium has been absorbed into the body, it is taken up by a number of cell types where it can be incorporated into selenoproteins. Selenoprotein P is quantitatively the main selenoprotein in plasma and is synthesised in the liver (Motsenbocker and Tappel, 1982). Within tissues, both the organic forms and inorganic forms of selenium are metabolised to a common active selenide intermediate. This selenide is then used to synthesise specific selenocysteine residues that are incorporated co-translationally into the active site of the selenoprotein. The main excretion pathway for selenium is through the urine, although excretion of a small amount occurs through the breath and skin (Daniels, 1996).

Selenium associated disorders

Selenium deficiency has been implicated in the pathogenesis of many diseases including Kashin-Beck disease and Keshan disease (Chen et al., 1980; Combs, 2001; Levander, 1987). Keshan disease had been described in populations inhabiting selenium-deficient areas of China. The disease is a multifocal myocarditis affecting children and females. Selenium supplementation of these communities has decreased the incidence of this disease but has not been able to reverse the heart failure in those who had already contracted the disease (Chen et al., 1980). Kashin-Beck disease is an endemic osteoarthropy affecting children and adolescent in selenium-deficient areas. Furthermore, selenium deficiency had been implicated in the pathophysiology of cancer, cardiovascular diseases, muscular dystrophy, and a number of other diseases.

Selenium toxicity can develop if selenium intake exceeds 800 µg/day (Kot and Namiesnik, 2000). The sign and symptoms of selenium toxicity include garlic-like breath, peripheral neuropathy, cirrhosis and traverse nail lines. However, other non-specific symptoms like gastrointestinal disturbance and fatigue are also associated with selenium toxicity.

Selenium Function

In normal physiology selenium regulates cellular growth and death and can modulate signal transduction in various cells (Greeder and Milner, 1980; Kim and Stadtman, 1997). Selenium can mimic insulin signalling restoring glycaemic control in diabetic mice. The exact mechanism for this action is yet fully understood. Most of the biological actions of selenium arise from its incorporation into the various selenoproteins. In addition, selenium has significant anti-inflammatory actions acting through a variety of mechanisms including modulation of the immune system.

Selenium and NAFLD

Selenium, acting through the synthesis of specific selenoproteins with powerful anti-oxidant actions, is able to prevent oxidative damage to cells. Since oxidative stress has been postulated as being second hit in the pathogenesis of NAFLD, it is of concern that the selenium intake in the UK falls well below that required to allow maximal expression of anti-oxidant selenoenzymes. Clearly the role of selenium in preventing hepatic damage and fibrosis associated with NASH warrants investigation particularly as there are no reports of such studies.

Selenoprotein synthesis

Selenoenzymes form an important battery of antioxidant enzymes that are extremely effective at preventing cellular damage that occurs through oxidative stress.
More than twenty five selenoproteins have been identified (Moghadaszadeh and Beggs, 2006). When selenium is incorporated into selenocysteine, it becomes an essential amino acid and is incorporated into the active site of the various selenoenzymes. Selenocysteine (Sec) is coded for the TGA/UGA codon. This UGA codon may be read as a stop codon if selenium supplement is limited. Therefore, it is essential that enough selenium is available to allow the full expression of selenoenzymes.

Selenocysteine (Sec) biosynthesis in mammals involves the incorporation of serine into SectRNA[ser]sec by seryl tRNA synthetase to yield SeryltRNA[ser]sec. The complex is then phosphorylated by phosphoseryl tRNA kinase. Selenium (selenide) is first phosphorylated by selenophosphate synthetase and then replaces the phosphate by the Sec synthetase. The resulting selenocysteyl-tRNA[ser]sec delivers the Sec into the growing polypeptide chain during translation (Papp et al., 2007) (see figure 1.5).

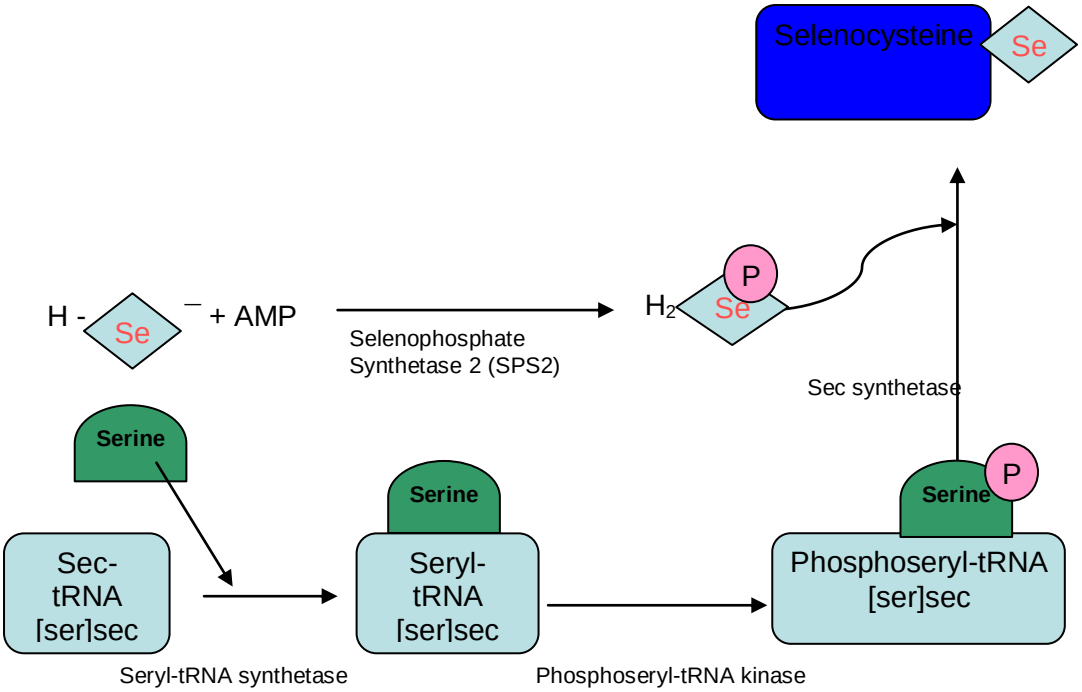


Figure 1.5: The mammalian synthesis of selenocysteine.

Sec: selenocysteine.

Although the availability of Sec is essential for the translation of the UGA codon into a proper selenoprotein, other factor involved in the process of translation should exist for the process to be successful. Such factors include selenocysteine insertion sequence (SECIS) element, elongation factor EFSec and SECIS binding protein 2 (SBP2) (Papp et al., 2007). Knockout of selenocysteine-t-RNA in mice has been shown to be lethal early in embryonic life (Bosl et al., 1997).

SECIS is the secondary RNA stem –loop structure. For the proper translation of selenoprotein, the coded mRNA should contain a SECIS element which is located in the 3' of the mRNA. The SECIS has a unique structure which contain an apical loop due to its consensus sequences that is distinctive for Sec incorporation (Papp et al., 2007).

Mammalian selenoproteins

The antioxidant properties of selenium are thought to be largely mediated through the expression of a variety of cytoprotective selenoenzymes that contain a selenocysteine residue in the active site. The family of glutathione peroxidases (GPX) and thioredoxin reductases (TR) are selenoenzymes that are considered particularly important for maintaining the antioxidant capacity of tissues. These families are represented in all tissues and the most important of these selenoenzymes for antioxidant function in tissue are thought to be cytoplasmic thioredoxin reductase (TR1), mitochondrial TR (TR2), cytoplasmic glutathione peroxidase (GPX1) and phospholipid hydroperoxide GPX (GPX4). The main anti-oxidant functions of these selenoenzymes are illustrated in figure 1.6.

Table 1.5: Mammalian selenoproteins and their main proposed roles.

Selenoprotein	Main role
Glutathione peroxidase	
GPX1	Antioxidant in cell cytoplasm, selenium reservoir?
GPX2	Antioxidant in the gastrointestinal tract
GPX3	Antioxidant in extracellular space and plasma
GPX4	Membrane antioxidant and structural protein in sperm
GPX5	Unknown
GPX6	Olfactory epithelium- and embryonic tissue-specific (Lu and Holmgren, 2009)
Thioredoxin reductase	Many functions including maintaing the redox state, detoxify peroxides and dithiol-disulphide oxoreductase
TR1	Cytosolic form
TR2	Mitochondrial form
TR3	Expressed mainly in the testes
Iodothyronine deiodinases	
Type 1 and 2	Converts T4 (inactive) to 3,5,3' T3 (active)
Type 3	Converts T4 (inactive) to 3,3,5' reverse T3 (active)
Selenoprotein P	Transport of selenium, antioxidant on endothelium
Selenoprotein W	Antioxidant in cardiac and skeletal muscle?
Selenophosphate synthetase (SPS2)	Synthesis of selenophosphate to form selenoprotein
15 kDa selenoprotein (Sep 15)	Protects against cancer (mechanism unknown?)
H,I,K,M,N,O,R,S,T,V	Mostly unknown

Adapted from (McKenzie et al., 2002).

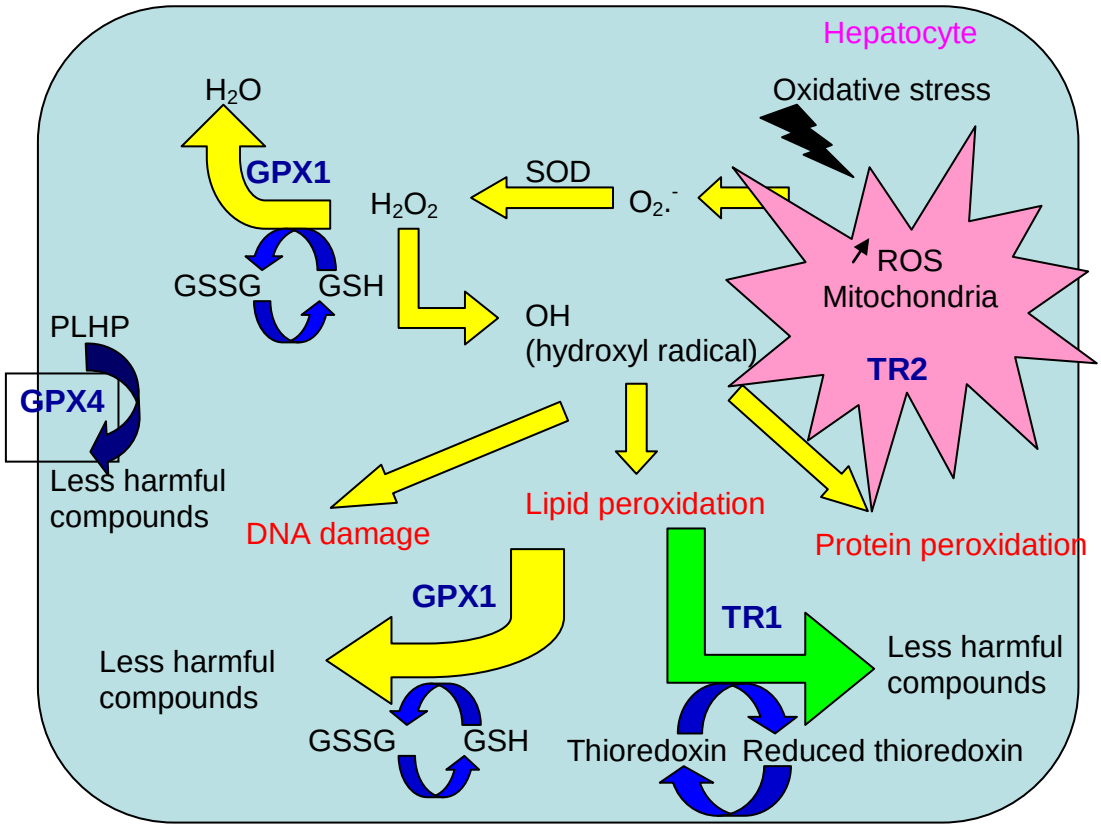


Figure 1.6: The role of the important selenoenzymes as anti-oxidants in oxidative stress. GPX1 and 4 (cytoplasmic and membrane glutathione peroxidase), TR1 and 2 (cytoplasmic and mitochondrial thioredoxin reductase), PLHP (phospholipid hydroperoxide), SOD (superoxide dismutase). Texts in bold blue indicate selenoenzymes.

Glutathione peroxidases

Glutathione is the substrate for all the GPXs and is used to reduce H_2O_2 and lipid peroxides to the corresponding alcohol (Nordberg and Arner, 2001). To date six GPXs have been described. GPX1 was the first selenoprotein to be discovered in the 1970s. Although distributed throughout the body, the liver, red blood cells and kidney have particularly high levels of GPX1 (Mates et al., 1999). This reflects the need of these tissues for GPX because they are particularly metabolically active and thus undergoing considerable oxidative stress. GPX1 is also known as a cytoplasmic GPX and it consists of four identical subunits. Each subunit has a binding site for glutathione and an active site containing a selenocysteine residue that is located in the N-terminal domain. GPX1 is known for its anti-oxidant activity acting through the reduction of many natural-occurring harmful compounds including hydrogen peroxide and fatty acid hydroperoxides (Flohé, 1989).

GPX 4 is the membrane-bound GPX that shares 45% homology with GPX1. It is monomeric and is involved in reducing the lipid hydroperoxides and cholesterol ester hydroperoxides within cell membranes (Mates et al., 1999). GPX2 and GPX3 are found mainly in the kidney and the intestines (Nordberg and Arner, 2001). GPX5 and 6 were recently discovered with GPX5 being structurally similar to GPX3 and GPX6 being similar to GPX4 (Fukuhara and Kageyama, 2005).

Thioredoxin reductases

The thioredoxin reductase family of selenoenzymes were discovered in 1996 (Holben and Smith, 1999). The thioredoxin reductases (TR1, TR2 and TR3) are FAD-containing homodimeric selenoenzymes that together with thioredoxin (Trx) as a substrate and NADPH as a cofactor, form a powerful dithiol-disulphide oxidoreductase system referred to as the TR/Trx system which is important in sensing and regulating the redox state of the cell (Mustacich and Powis, 2000). TR1 is found in the cell cytosol of all tissues while TR2 is found in the mitochondria (Kohrle et al., 2005). TR2 is considered to be important in maintaining the redox balance in the most important ‘factory’ of ROS production, namely the mitochondria,

and hence the role of TR2 may be to protect the mitochondria and thereby preserve cell viability. These enzymes are expressed at high levels in liver, kidney and the heart (Kohrle et al., 2005). TR3 is a variant of TR2 but solely expressed in the testis (Su et al., 2005). In addition to their antioxidant roles, mammalian TRs can catalyse the reduction of a variety of substrates including thioredoxin, protein disulphide-isomerase, low molecular weight disulphides such as 5,5'-dithiobis-2-nitrobenzoic acid, lipoic acid, vitamin K and dehydroascorbic acid.

The TR/Trx system has been associated with many diverse cellular functions including the regulation of cell growth, inhibition of apoptosis (Arner and Holmgren, 2000) and regeneration of proteins inactivated by oxidative stress. In addition, TR1 can also act directly to detoxify hydrogen peroxide and a wide variety of lipid hydroperoxides (Brigelius-Flohe et al., 2003). Selective inhibition studies suggest that TR1 may be more important than the glutathione peroxidases (GPX) in protecting human cells against oxidative damage. Modification of hydrogen peroxide signalling should be possible by manipulating the expression of GPX and TR through changes in selenium status.

Regulation of selenoprotein expression and the hierarchy of selenoproteins

The selenoprotein expression in cells and between tissues is highly regulated through a strict "hierarchy" of selenoprotein expression (Driscoll and Copeland, 2003). This regulation is dependent on the availability of selenium (Behne et al., 1988; Bermano et al., 1996; Burk and Hill, 1993; Hill et al., 1997a; Weitzel et al., 1990). However, this availability does not affect the transcription rate of any of the selenoprotein genes (Heider et al., 1992; Hirota et al., 1997; Howie et al., 1995) but it affects the mRNA levels which is related to mRNA translation and/or stability (Heider et al., 1992; Howie et al., 1998). Several other factors besides selenium supply can influence the expression of specific selenoproteins. For example oxidative stress could lead to induction of TR and GPX expression (Brigelius-Flohe et al., 1997). Also, in a tissue specific manner, activation of second-messenger pathways could modifies the expression of specific selenoproteins (Karin et al., 1997).

The availability of selenium is reflected in the differential expression of the various selenoproteins and changes in selenium status affect the expression of selenoproteins in a tissue-specific manner (Behne et al., 1988; Bermano et al., 1996; Burk and Hill, 1993; Hill et al., 1997a; Weitzel et al., 1990). During selenium deficiency, a hierarchy mechanism ensures that selenium and selenoprotein expression is retained in some tissues at the expense of others. In addition within an individual tissue there is also a hierarchy of selenoprotein expression; for example in selenium deficiency GPX-1 expression is rapidly lost whilst TR2 expression is maintained. Examples of tissues that readily release their available selenium to other organs in cases of selenium deficiency are liver, muscle and kidney. The brain, reproductive and endocrine systems seem to have an essential requirement for selenium at all times so they have priority in accessing selenium in selenium-deficient situations (Behne et al., 1988; Bermano et al., 1995).

TR1 expression is retained in selenium deficiency to a greater extent than GPX1 (Berggren et al., 1999; Gasdaska et al., 1999). *In vitro* studies suggest that selenium can stabilize the mRNA of TR1 leading to increased synthesis of the active enzyme (Gallegos et al., 1997). Indeed some have suggested that the primary role of GPX-1 is to act a buffer to hold a reservoir of selenium within cells that can be called upon to express other more important selenoproteins at times of selenium deficiency (Bermano et al., 1995).

1.1.4 Future Therapies

Because the pathophysiology of liver fibrosis involves various cellular injury mechanisms, it has been argued that prospective therapy should be constructed to target all of these various mechanisms (Li and Friedman, 1999). This approach includes therapies that:

1. alleviates the injurious factor
2. inhibits the inflammatory process
3. inhibits the activation of hepatic stellate cells
4. lyses the matrix formed

Alleviating the injurious factor is an appropriate way of approaching the treatment of any disease. Secondary NAFLD could benefit from this strategy, where the cause is identifiable and treatable. In the case of primary NAFLD, the complex interrelationship between insulin resistance, inflammation, fat-loading and oxidative stress in the pathogenesis of the disease means that deriving combined therapies to tackle each of these metabolic problems is problematic (Li and Friedman, 1999).

As inflammation is a major contributor in the process of fibrogenesis, the use of anti-inflammatory therapies should have potential benefit. Corticosteroids have been used widely to decrease inflammation in many diseases including liver fibrosis (Tanner and Powell, 1979). Autoimmune hepatitis has benefited from corticosteroid treatment more than any other type of liver fibrosis (Mathurin et al., 1996). Several other anti-inflammatory agents had been used, including ursodeoxycholic acid (Corpechot et al., 2000), captopril (Tuncer et al., 2003) and angiotensin-converting enzyme (ACE) inhibitors (Julie et al., 2001). All of these anti-inflammatory agents have been proven to decrease liver fibrosis in animal models.

Because stellate cells are considered the major contributor to the fibrogenic process in NAFLD, inhibiting the activation of these cells would theoretically also be one attractive therapeutic approach to the clinical problem. Although, the relationship between oxidative stress and hepatic stellate cells is not yet clear, some studies claim that direct oxidative stress on the stellate cells could be an effective way to activate

them and thus contribute to fibrogenesis (Apte, 2002). However most studies support the view that stellate cell activation depends on factors released by nearby injured cells such as hepatocytes and Kupffer cells that have been damaged by chronic oxidative stress. These damaged cells then release various growth factors and inflammatory cytokines that have a major role in the activation of hepatic stellate cells. This proposed relationship between oxidative stress and hepatic stellate cells is considered by some to be the most important mechanism for stellate cell activation (Friedman, 1993). Clearly decreasing oxidative stress, either directly or indirectly, should theoretically be advantageous in preventing the activation of hepatic stellate cells. For this reason, many studies have investigated the ability of anti-oxidants to prevent stellate cells activation.

A number of anti-oxidants had been investigated but whilst some studies have showed promising results others have failed to show a beneficial effect of antioxidants. The anti-oxidants studied include vitamin E (Parkkila et al., 1996), vitamin C (Helen and Vijayammal, 1997), silymarin (Hosny et al., 2007), and many others. Certainly, the antioxidants dilinoleoylphosphatidylcholine and S-adenosylmethionine have antifibrogenic actions in LX-2 cells (Cao et al., 2006).

Table 1.4: Various anti-oxidants trials used in the hepatic fibrosis treatment.

Anti-oxidants	**Reference**
Retinoids	(Okuno et al., 1990)
Vitamin E	(Parkkila et al., 1996)
Polyenylphosphatidylcholine (PPC)	(Ma et al., 1996)
Vitamin C	(Helen and Vijayammal, 1997)
S-adenosyl methionine	(Lieber, 2002)
Sylimarin	(Hosny et al., 2007)

The role of selenium and selenoproteins as antioxidants used to treat NAFLD has not been addressed (see below)

Various signalling cytokines have been described to play a role in the activation of stellate cells. Of these, the most important are PDGF and TGF-β1. Anti-sense mRNA of PDGF has been successful in reducing the fibrogenesis progression in an animal model (Borkham-Kamphorst et al., 2004). Inhibiting the signalling of TGF-β1 in stellate cells using different strategies was also beneficial in reducing the fibrogenesis process. The use of anti-sense mRNA, inhibition of TGF-β1 receptor phosphorylation and inhibition of intracellular Smad7 signalling pathways seems also to be beneficial (Liu et al., 2006).

Treatments designed to promote the degradation of the scar matrix have also been reported. The administration of antibodies against tissue inhibitor of metalloproteinase-1 (TIMP-1) was effective in breaking down the fibrosis *in vivo* (rats) (Parsons et al., 2004). However, therapies designed to attack the extracellular matrix should be selective to degrade the collagen laid down in fibrosis (type I and III), and not the "normal" physiological collagen that is essential to maintain the normal extracellular matrix (i.e. collagen type IV) (Friedman, 1993). Unfortunately, to date, no study has been able to identify an agent that distinguishes between normal and abnormal fibrosis.

1.2 *In-vivo* and *in-vitro* models of NAFLD:

A number of research methods to investigate the pathogenesis of NAFLD have been used.

***In-vivo* models of NAFLD**

The use of animal in-vivo models to investigate liver fibrosis has several advantages. Firstly it provides a complementary picture of the pathophysiology when various systems such as the immunological and endocrine systems integrate in the fibrotic process. Animal models also often provide a good way of studying the whole picture of cell to cell communications. Drawbacks to animal models include ethical issues and technical difficulties (e.g., mice have a small blood volume) as well as concerns the results from such models do not necessarily accurately reflect the human pathophysiology.

Various animal models had been used to reflect different types of fibrosis. Depending on the cause of fibrosis, animals have been subjected to different injurious agents to mimic the human cause of fibrosis. Of these models the most commonly used include induction of chronic injury and fibrosis by the administration of different toxic agents such as carbon tetrachloride (Pierce et al., 1987), thioacetamide (Muller et al., 1988), dimethylnitrosamine (George et al., 2001), and ethanol (French, 2001). The administration of heavy metals such as copper (Toyokuni et al., 1989) and the ligation of the common bile duct (Kountouras et al., 1984) have been also used to mimic Wilson's disease and cholestatic causes of human fibrosis, respectively.

Recently, the administration of a methionine/choline-deficient diet was found to mimic the pathophysiology of NAFLD whereby fat-loading, inflammation and fibrosis were observed in the animal model (George et al., 2003; Rizki et al., 2006). In addition, several attempts to develop transgenic animal models to study the various genes involved in the process of fibrogenesis have been reported (Arias et al., 2003).

In-vitro models of NAFLD

The commonly employed *in-vitro* research models are based either on the use of primary cells of hepatocytes and hepatic stellate cells or the use of immortalized cell lines.

Primary cells

Although, the use of primary cells still retaining most of their *in-vivo* characteristics, would appear ideal for the study and further dissection of the pathophysiology process, their availability (especially in the case of human cells), relatively short life, and the time required to isolate and harvest such cells make them less attractive for use in the research laboratory. Furthermore these primary cells can spontaneously transform to a different phenotype in culture (Friedman et al., 1989).

Cell lines

Heaptocyte cells lines from primary human or rodent liver cells have been developed. Genetically modified cell lines have been developed by the transfection of the large T antigen of simian virus 40 or polyoma virus (Xu et al., 2005) into primary cells. Another method aimed to extend the life span of primary cells is the transfection of cells with telomerase reverse transcriptase (Schnabl et al., 2002). The advantages of cell lines include their wide availability, their simplicity of use and that they allow a molecular dissection of the pathophysiological processes occurring in fibrogenesis.

LX-2 cell line

All hepatic stellate cell lines that had been culture in uncoated plastic are of an activated phenotype (Xu et al., 2005). Recently used human stellate cells lines include the LX-1 and LX-2 lines which have been very well characterized (Xu et al., 2005).

Because LX-2 cell line has had a better similarity to primary activated stellate cells than LX-1 cell line, LX-2 cell line was chosen in our study to represent an activated hepatic stellate cells.

The human LX-2 stellate cell line expresses key receptors regulating hepatic fibrosis, including platelet-derived growth factor receptor β (βPDGF-R), obese receptor long form (Ob-R_L), and discoidin domain receptor 2 (DDR2). LX-2 cells also express proteins involved in matrix remodelling; matrix metalloproteinase (MMP)-2 and tissue inhibitor of matrix metalloproteinase (TIMP)-2. LX-2 proliferates in response to TGF-β1 and express mRNAs for α-1 pro-collagen and HSP47 (Xu et al., 2005). Transforming growth factor β1 gene expression was found to be induced in these cells. LX-2 cells have a retinoid phenotype typical of stellate cells. Microarray analyses show there is a strong similarity in gene expression between primary HSCs and LX-2 (98.7%), with expression of multiple neuronal genes (Xu et al., 2005). LX-2 cells are widely regarded as a suitable cell line to investigate the activation process and its relation to the pathophysiology of fibrosis.

C3A cell line

The C3A cell line is a specialized hepatocyte cell line derived from the human hepatoblastoma cell line HepG2. Although, there is considerable literature regarding HEPG2 cells as a suitable model to study hepatocyte function and its responses to injurious factors during liver fibrosis, the C3A cell line appears even more physiologically functional than the HepG2 (Filippi et al., 2004; Mavri-Damelin et al., 2008).

Studies on extrahepatic bioartificial liver devices have used the C3A cell line because of its ability to perform most of the primary hepatocyte functions including albumin production, detoxification of toxins via cytochrome P450 systems and production of urea. These functions were found to be either lost or diminished in HepG2 in comparison to C3A cells. The C3A cell line is found also to have a comparable nitrogen metabolism to perfused rat liver (ATCC). Table 1.5 illustrate the functions of HepG2 and C3A cell lines compared to the primary cell line.

Furthermore, one study notes that C3A cells when cultured in 3-dimensional spheroids shows a more typical morphology of the primary hepatocyte i.e. tight junctions, polar cells and bile caniculi and moreover, enhanced cytochrome P450 expression than in the conventional culturing of this cell line (Elkayam et al., 2006). In contrast to C3A cells, primary hepatocytes had been found to lose their metabolic activity and more importantly, cease proliferation relatively quickly.

One study showed further enhancement of the functionality of the C3A cell line through conditioning the cells with lactate/ pyruvate, octanoate and ammonium chloride (LPON). With this treatment, gluconeogenesis, β-oxidation and urea production were all increased (Filippi et al., 2004).

Given the above information, we decided that the C3A cell line would be an appropriate model to use for the studies in this thesis.

Property	**Hepatocyte primary cell**	**C3A cell line**	**HepG2 cell line**
Albumin production	***	**	*
Alpha fetoprotein production	***	**	*
Grow in glucose deficient medium	*	**	*
Cytochrome P450	***	**	*
Urea production	***	**	*

Table 1.5: Comparison of the main properties of hepatocyte primary cell to the C3A and HepG2 cell lines

1.3 Project Aims:

The aims of this thesis were to address the following questions:

1. Can C3A and LX-2 cell lines be cultured in a selenium-deficient medium that allows cell viability to be retained and allow study of the expression of the two main cytoplasmic selenoenzymes (TR1 and GPX1) that can perform the anti-oxidant action in these cells? (Chapter three)
2. Do treatments with fatty acids lead to fat-loading of C3A and LX-2 cells?. Do such treatments modify the expression of TR1 and GPX1? (Chapter four)
3. Does selenium supplementation of C3A and LX-2 cells protect them from oxidative damage induced by lipid hydroperoxides? (Chapter five)
4. What is the relationship between α-1 pro-collagen mRNA expression and selenium status in LX-2 and C3A cells? (Chapter six)
5. What is the selenium status of healthy subjects in the UK?. Does this differ from obese subjects who have developed insulin resistance (type 2 diabetes)? (Chapter seven)

2 CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 *Materials*

2.1.1 Commercially available material

2.1.1.1 Cell culture materials

Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose); Hank's balanced salt saline (HBSS); phosphate-buffered saline (PBS); sodium pyruvate; L-glutamine; penicillin/streptomycin solution; amphotericin B solution; trypsin; fetal bovine serum (FBS) were purchased from Lonza Ltd. (Wokingham,, UK).

2.1.1.2 Radioisotopes

For protein iodination of TR1 and TR2, Bolton & Hunter reagent (18.5 MBq, 500 µCi) and iodine-125 (37 MBq, 1 mCi, specific radioactivity 16 MBq/ nmol) were supplied by GE Healthcare, Little Chalfont, UK.

2.1.1.3 Other chemicals

Acetic acid; ethanol; ethylenediaminetetraacetic acid (EDTA); hydrochloric acid; methanol; microcrystalline cellulose; orthophosphoric acid; nitric acid; transforming growth factor β1 were supplied by MERCK, Leicester, UK.

Bovine serum albumin powder (BSA); Coomassie brilliant blue (R-250 and G-250); 5,5′-dithiobis (2-nitrobenzoate) (DTNB); dithiothreitol (DTT); insulin; G-100 gel for gel filtration; glutathione; glutathione reductase; fatty acid-free bovine serum albumin; NADPH; sodium azide; sodium selenite; Sephadex G-75 gel; thioredoxin; t-butyl hydroperoxide, menadione, and hydrogen peroxide were supplied by Sigma

Aldrich Company Ltd, Poole, Dorset, UK. Lactate dehydrogenase (LDH) kits, Triglyceride (TG) kits and Cholesterol kits were purchased from Alpha Laboratories (Eastleigh, Hampshire, UK). Precinorm®U Universal control serum was supplied by Boehringer (Ingelheim, Germany).

Human α-1 pro-collagen primers and probe were purchased from Eurofins MWG operon (London, UK):
Forward primer 5'-
CAAGAGGAAGGCCAAGTCGAGG-3',
Reverse primer 5'-
CGTTGTCGCAGACGCAGAT-3' and
Probe FAM 5'-
CCTCAGGTACCATGACCGAGACGTGTGGAAACC-3' TAMRA.
All materials needed for RNA extraction, cDNA and Taqman^{TM} steps were purchased from Applied Biosystems (Warrington, UK).

Thioredoxin reductase 1 (TR1) and 2 (TR2) proteins were obtained from Lab Frontier (Seoul, Korea). Sheep anti-rabbit serum (secondary antibody used in TR1 and 2 RIA) were purchased from (Fitzgerald laboratories, concord, USA). The Cytometric Beads Array (CBA) was purchased from BD Bioscience, San Diego, CA, USA.

Cell culture inserts for 6-well plates (3.0 µm, translucent polyethylene terephthalate (PET) membrane) were purchased from BD Falcon, BD Bioscience, San Jose, CA, USA.

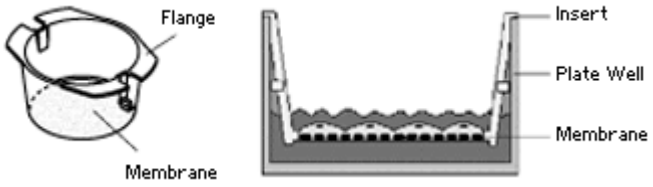


Figure 2.1: Illustration of the cell culture inserts (left) and its application on the well (right). (BD bioscience website http://www.bdbiosciences.com/discovery_labware/technical_resources/cellculture.shtml))

2.1.2 Non-commercially sourced materials

2.1.2.1 Cell lines

The LX-2 cell line was a kind gift from Dr Scott Friedman (Division of Liver Diseases, Mount Sinai School of Medicine, New York, USA). The C3A cell line was a gift from the Hepatology Unit, the Royal Infirmary of Edinburgh.

2.1.2.2 Blood samples

For data in chapter seven, blood samples were collected from patients with type 2 diabetes mellitus under the supervision of Prof. Peter Hayes from both the liver unit and diabetic clinics. The study was approved by the Ethics committee of Edinburgh Royal Infirmary, NHS Lothian.

2.1.2.3 Antibodies

Antibodies to human placental thioredoxin reductase (TR1) were raised in rabbits to cytosolic proteins purified to homogeneity. Dr Forbes Howie, Clinical Biochemistry, University of Edinburgh supplied the antibodies to human TR1. TR2 antibody was kindly supplied by Professor John Arthur, Rowett Research Institute, Bucksburn, Aberdeen, UK.

2.2 *General methods*

2.2.1 Cell culture and maintenance

C3A and LX-2 cell lines were grown in 75-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (v/v), sodium pyruvate (1 mM), 2 mM L-glutamine, 100 IU penicillin, 100 mg/ml streptomycin and amphotericin B (0.25 µg/ml) and incubated at 37 °C in a 5% CO_2-air humidified atmosphere. The medium was changed 48 hr after initial plating. Cells were passaged every 7-10 days.

2.2.2 Cell collection

Cells were collected, unless otherwise stated as follows: Cell monolayers were washed twice with 2 ml HBSS, and harvested via scraping with 2 ml HBSS. Efficiency of harvesting was determined by light microscopic inspection. The cells were then pelleted by centrifugation at 500 x g for 10 min. The HBSS was aspirated, and the pellets frozen at - 80°C until enzyme assays were carried out. Prior to assessment, the cell pellets were thawed resuspended in 200 µl and lysed by sonication (three pulses of 10 sec using a Soniprep 150 Sonicator) on ice in 0.125 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1 % Triton X-100 (peroxide- and carbonyl-free).

2.2.3 Enzyme activity assays

2.2.3.1 Thioredoxin reductase activity assay

This assay was performed on a Cobas-Fara centrifugal analyser (Roche Diagnostics, UK) by adapting the method of (Hill et al., 1997b)

The assay reagent comprised of 5 mM DTNB, 0.24 mM NADPH, 10 mM EDTA, 0.2 mg/ml BSA in 100 mM potassium phosphate/50 mM potassium chloride buffer, pH 7.0. EDTA is necessary since mammalian Thioredoxin reductase activity is inhibited by heavy metal ions. Assay reagent was stored in the dark when not in use.

A placental cytosol preparation was used as a control: Normal 40-wk full-term placentas obtained within 2 hr of delivery were used, and all procedures were performed at 4°C or on ice. The placenta (~550 g) was cut into pieces 4 cm^3. Following removal of the umbilical cord and amniotic membrane, the pieces of placenta were washed with 2 L of 50 mM Tris/HCl, pH 7.4, at room temperature, containing 1 mM EDTA, 0.5 mM DTT (buffer A), and centrifuged at 30,000 *x g* for 1 hr.

Four internal controls were prepared by diluting a pooled stock solution of human placental cytosol with FBS at two-fold, four-fold, sixteen-fold and thirty two-fold dilutions. The controls were constructed to cover the range of activities given by the different cell types in the assay, as assessed by historical lab data (~ 0.25 to 5.0 U/g proteins). The controls were dispensed into 180 µl aliquots and frozen at - 80°C. Good linearity was demonstrated for increasing dilutions of human placental cytosol.

Cell pellets were collected as described in section 2.2.2. The lysates were centrifuged at 500 x g, and kept on ice until assay took place.

This assay is based on the conversion of the substrate DTNB (5, 5´- dithiobis 2-nitrobenzoate) to a yellow chromophore (absorption max at 412 nm), according to the reaction:

DTNB + NADPH + H^+ → 2 TNB (thiolate thionitrobenzoate) + $NADP^+$

Thioredoxin reductase activity was measured on a Cobas-Fara centrifugal analyser (Roche Diagnostics, UK) using 30 µl of sample pipetted into the cuvette ring with 30 µl diluent (distilled H_2O) and 190 µl of reagent. All samples were warmed to 37°C prior to assay. The assay was performed on the Cobas-Fara centrifugal analyser with absorbance measurement at 412 nm over a period of 0.5 to 290.5 seconds. The absorbance was read once every 10 seconds following an initial reading at 0.5 sec, and the rate of absorbance change determined by kinetic analysis over the linear portion of the curve (100.5 to 250.5 seconds). Results were corrected for protein measured by the Bradford method (section 2.2.5) using BSA as standard.

The extinction coefficient of TNB at 412 nm is 13.6 mM^{-1} cm^{-1} (Holmgren and Bjornstedt, 1995). TR activity is expressed in units per gram protein (U/g). One unit of mammalian TR activity is defined as 1 μmol TNB formed per minute (Holmgren and Bjornstedt, 1995).

Intra-assay and inter-assay precision data as previously calculated by Dr Michelle Lewin (Lewin, 2003) using four quality control (QC) pools in one assay were ranging from 5-18 % and 17.1-21.4 % respectively.

2.2.3.2 Glutathione peroxidase activity assay

The reagent was made up of the following: 40mM NADPH, 60 mg reduced glutathione, 28 U glutathione reductase, 28 ml of assay buffer which is comprised of (0.05 M) sodium phosphate buffer (pH 7.4), 5 mM EDTA and 1 ml sodium azide (112.5 mM). Hydrogen peroxide reagent was prepared separately (0.077 mM). The reaction mix was placed in a water bath at 25°C to achieve temperature equilibration.

Control blood samples obtained from Blood Transfusion Unit (Royal Infirmary of Edinburgh) were diluted 20-fold before use.

GPX1 activity was measured using an assay method adapted for use on the Cobas-Fara centrifugal analyser. The method used was as previously described in our laboratory (Beckett et al., 1990), and was based on the method described by DE Paglia and WN Valentine (1967). Briefly, GPX1 activity is assessed by monitoring the rate of conversion of NADPH to $NADP^+$, measured as a change in absorbance at 340 nm in the presence of the substrate hydrogen peroxide (detailed in the reaction equation below).

The reaction catalysed by GPX1 and utilised for its assay is as follows:

2GSH (reduced glutathione) + H_2O_2 → GSSG (oxidised glutathione) + $2H_2O$

Glutathione reductase is added to the reaction mix to maintain a constant concentration of glutathione.

To measure GPX1 activity on the Cobas-Fara centrifugal analyser, a 20 µl sample was automatically pipetted along with 40 µl diluent (distilled H_2O) and 183 µl reaction mix into a cuvette. The blank rate with water in place of the substrate was also measured. The reaction was started by addition of 7 µl of the hydrogen peroxide substrate solution, and the subsequent reaction rate was followed for 120 sec at 340 nm. The first reading was taken at 0.5 sec, with each subsequent reading at 5 sec intervals.

A unit of GPX1 is defined as that which oxidises 1 µmole of NADPH per min. The molar extinction coefficient of NADPH is 6200 M^{-1} cm^{-1}. The blank rate was subtracted from the rate obtained after substrate addition.

2.2.4 **Lactate dehydrogenase activity assay (LDH)**

Cell viability was assessed as the percentage retention of lactate dehydrogenase (LDH) by the cell. This assay is based on the procedure of Gay et al (1968). Briefly, intracellular and extracellular LDH activities of the cell lysates and culture medium respectively, were determined by following the rate at which NAD^+ is reduced to NADH measured as a decrease in absorbance at 340 nm in the presence of lactate using a LDH kit method (Alpha Laboratories, Eastleigh, Hampshire, UK) modified for use on the Cobas-Fara centrifugal analyser. The rate of decrease in absorbance at 340 nm, measured at 37°C, is directly proportional to LDH activity in the sample. The results were expressed as % LDH activity retained, calculated as follows: intracellular LDH activity/ (extracellular LDH activity + intracellular LDH activity) x 100.

Reagents were reconstituted by the addition of 10 ml of deionized water as indicated on the vial. After addition of water, the vial was stoppered and mixed by gentle inversion several times. The vial active ingredients were:

Tris buffer (100 mM), NAD (7 mM), lithium lactate (50 mM) and KCl (120 mM).

For cell viability studies all cells were plated into 12 well plates at a density of 1 x 10^5 cells/ml for C3A cells and 0.5 x 10^5 cells/ml for LX-2 cells. After treatment with t-BuOOH, 1 ml of the culture medium was removed from the cells and placed into pre-labelled Eppendorf tubes. Cell pellets were collected as described in section 2.2.2. Cell debris in the culture medium and cell lysates were removed by micro-centrifugation of all samples at 11,000 *x g* for 10 min prior to assay for LDH activity.

Precinorm® U (a commercially produced 'universal control serum; Boehringer (Ingelheim, Germany)) was used for quality control for every assay run.

The change in absorbance at a wavelength of 340 nm was measured in 25 µl of sample in a final volume of 1500 µl of the reagent. The sample and reagent were pipetted into the cuvettes and incubated at 37°C for 30 sec. The change in absorbance of the reaction mixture was read at 0.5 sec, and was then read every 5 sec for a total of 20 readings. One unit of activity per litre was calculated as follows:
Activity in U/l=Δ absorbance/min×factor
where the factor = total reaction volume (ml)×1000/6.3×sample volume (ml)×cuvette pathlength (cm)
The LDH activity was expressed as units per litre (U/l) as determined by kinetic analysis.

The LDH activity was also measured in culture medium that had not been in contact with cells as a measure of endogenous LDH in the culture medium, as well as after addition of Triton X-100 and PBS (lysis conditions). These blank values were subsequently subtracted from each extracellular and intracellular LDH measurement. The LDH activities in both the media and cell lysates were then calculated as described above.

According to the manufacturer, the inter-assay CV (%) is 3.2 and the intra-assay CV (%) is 2.3.

2.2.5 Bradford assay for protein measurements

Protein measurements were carried out utilising the dye-binding assay of Bradford (Bradford, 1976) adapted for use on the Cobas-Fara centrifugal analyser.

The Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. Then, 100 ml 85 % (w/v) phosphoric acid was added, and the mixture stirred for 30 min. The resulting solution was diluted with distilled H_2O to a final volume of 1000 ml, filtered through Whatman grade 1 filter paper and stored at room temperature in a closed bottle.

A standard curve was constructed using bovine serum albumin (BSA) as standard, and distilled water as the diluent, covering the range 0 to 100 mg/l.

Bradford reagent (256 µl) was added to each cuvette, which was incubated for 100 sec at 37°C prior to sample addition, with an initial absorbance reading (595 nm) taken at 95 sec. Following the addition of 25 µl of sample plus 50 µl distilled H_2O (diluent) to the cuvettes, a further incubation took place for 180 sec at 37°C. A final absorbance was then read at 595 nm. The difference between the final and initial absorbencies was calculated, and a standard curve plotted.

The protein concentrations of samples were interpolated from the standard curve. Samples were diluted with distilled water to fall in the middle portion of the standard curve and a quality control (QC) was run with every rotor to assess the reproducibility of the results.

Cells were collected as described in section 2.2.2 and then diluted 50 times with distilled water.

2.2.6 Triglyceride measurements

Triglyceride measurements were made using a commercial kit adapted for use on the Cobas-Fara centrifugal analyser. The triglyceride assay is based on the method of Wako and the modifications by (McGowan et al., 1983) and P Fossati et al. (1982). The assay runs according to the following reactions:

$TG + H_2O \rightarrow$ Glycerol + free fatty acids

Glycerol + ATP → Glycerol -3 phosphate + ADP

Glycerol-3-phosphate + O_2 → DAP + H_2O_2

H_2O_2 + 4-AAP + 3,5 DHBS →Quinone-imine dye + $2H_2O$

In reaction (a), triglycerides are converted to glycerol and fatty acids in the presence of water (H2O); in reaction (b), glycerol in the presence of adenosine triphosphate (ATP) is converted to glycerol -3 phosphate and adenosine diphosphate (ADP) while In reaction (c), glycerol -3 phosphate is oxidised by oxygen (O2) to form dihydroxyacetone phosphate (DAP) and hydrogen peroxide (H2O2). In the final step (d), the hydrogen peroxide (H_2O_2), 4-aminoantipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzene sulfonate (DHBS) are converted to a red coloured dye. The concentrations of triglycerides are proportional to the dye absorbance.

The ready-to-use reagent contained the following ingredients:
ATP (2.5 mM), Mg acetate (2.5 mM), 4-aminoantipyrine (0.8 mM), DHBS (1 mM), glycerol phosphate oxidase (GPO) (microbial) (>3000U/l), glycerol kinase (microbial) (>100 U/l), lipoprotein lipase (microbial) (>2000 U/l), horseradish peroxidase (>300 U/l), buffer (53 mM, pH 7).

Cell pellets were treated as described in section 2.2.2. Cell debris in the cell lysates were removed by micro-centrifugation of all samples at 11,000 x *g* for 10 min prior to assay for triglyceride measurement.

Precinorm® U was again used for quality control for every assay run.
Samples (100 µl) were transferred into Cobas cups for triglyceride measurement. The change in absorbance at a wavelength of 500 nm was measured in 3 µl of sample in a

final volume of 303 µl (300 µl reagent and 3 µl sample). The sample and the reagent were pipetted into the cuvettes and incubated at 37°C for 300 sec. The triglyceride concentration was expressed as mM as determined by kinetic analysis.

According to the manufacturer, the total CV (%) is 4.5 and the intra-assay CV (%) is 0.5.

2.2.7 Cholesterol measurements

Cholesterol measurements were accomplished by using a commercial kit adapted for use on the Cobas-Fara centrifugal analyser. The cholesterol assay is based on enzymatic oxidation of cholesterol using cholesterol oxidase after hydrolysis of cholesterol esters. The formulation was based on the method of (Allain et al., 1974) as modified by (Roeschlau et al., 1974).

The assay proceeds according to the following reactions:
Cholesterol esters → Cholesterol + Fatty acids
Cholesterol + O_2 → Cholest-4-en-3-one + H_2O_2
$2H_2O_2$ + Hydroxybenzoic acid + 4-aminoantipyrine → Quinoneimine Dye +4 H_2O
The concentrations of cholesterol are proportional to the dye absorbance.

The reagent was obtained ready to use and contained the following ingredients: Cholesterol oxidase (>200 U/l), cholesterol esterase (>500 U/l), horseradish peroxidase (>300 U/l), 4-aminoantipyrine (0.25 mM), hydroxybenzoic acid (10 mM), buffer (50 mM, pH 6.7) and surfactants.

Cell pellets were collected as described in section 2.2.2.

Precinorm® U was used for quality control in every assay run.

Samples (100 µl) were transferred into Cobas cups for cholesterol measurement. The change in absorbance at the wavelength of 500 nm was measured in 3 µl of sample in a final volume of 303 µl (300 µl reagent and 3 µl sample). The sample and the reagent were pipetted into the cuvettes and incubated at 37°C for 300 sec. The cholesterol concentration was expressed as mM as determined by kinetic analysis.

According to the manufacturer, the total CV (%) is 2.8 and the intra-assay CV (%) is 2.8.

2.2.8 Thioredoxin reductase 1 radioimmunoassay (RIA)

2.2.8.1 Introduction

Thioredoxin reductase1 (TR1) was measured using an 'in-house' radioimmunoassay developed in this laboratory by Dr. Forbes Howie, and has been described previously (Lewin et al., 2001; Miller et al., 2001).

2.2.8.2 Preparation of antibody

Primary antibody was raised in rabbits to an immunogen of human TR1 purified from placenta. The pre-precipitated secondary antibody was prepared by adding 25 ml of sheep anti-rabbit serum to 1.5 ml normal rabbit serum and mixing overnight at room temperature to precipitate out the immunoglobulins. Following centrifugation (230 *x g*, 5 min), the supernatant was discarded and the precipitate washed four times with 0.05 M phosphate buffer (pH 7.4) containing 0.1 % BSA and 0.02 % sodium azide. After the final wash the supernatant was removed and the pellet was resuspended in the same 0.05 M phosphate buffer as described above, to give a final volume of 100 ml.

2.2.8.3 Preparation of the assay diluent

The assay was performed using a diluent of 25 mM potassium phosphate buffer, pH 7.5, containing BSA 1 g/l, NaN_3 0.2 g/l, and 10 mM dithiothreitol. This diluent was used for both the antibody and the tracer.

2.2.8.4 Preparation of ^{125}I-TR tracer

Purified human placental TR1 was iodinated by Dr Forbes Howie using the Bolton-Hunter iodination procedure (Bolton and Hunter, 1973). Briefly, the benzene from the Bolton Hunter reagent was dried off by directing a gentle stream of dry nitrogen into the vial (fume hood). Pure TR1 (5 μg) in borate buffer was then added. Then the mixture was vortexed and spun down briefly. After incubation on ice for 60 min, 0.3 ml borate/glycine buffer was added to stop the reaction. The tube then left for 10 min on ice. After that, 0.3 ml phosphate/BSA buffer was added and mixed. The mixture was then applied to a Packed 10 ml glass column of swollen Sephadex G-75 gel, with H_2O plus 0.02% sodium azide and equilibrated with phosphate buffer, pH 7.4.

The column was then washed with 0.5 ml of Phosphate buffer before connecting a wash reservoir. After that, Every 1 ml fraction was collected separately until 25 ml were available.
Radioactivity of these fractions was measured and fractions in the first peak were pooled. The ^{125}I-TR tracer was immediately diluted 1:2 with FBS and stored at -20°C for up to three months. For use in the radioimmunoassay the ^{125}I-TR tracer was diluted with assay diluent so that approximately 10,000 cpm was added to each tube (mass approximately 50-100 pg TR/ tube).

2.2.8.5 Preparation of standards, controls and samples

Standards were prepared by diluting a stock solution of purified placental human TR1 (1 mg TR/l) with FBS to give the following concentrations: 0.5, 1, 2, 5, 10, 25, 50 µg TR/l. Three controls were made by diluting a stock solution of human placental cytosol with FBS to give values of approximately 2.5, 5 and 25 µg TR/l. Both standards and controls were dispensed into aliquots and frozen at - 80°C.

Cell pellets were stored at - 80°C immediately following harvesting. Prior to assay, samples were removed from the freezer, thawed, lysed by sonication, centrifuged and kept on ice until assay took place. Each sample was diluted with radioimmunoassay buffer between 1:10 and 1:25.

2.2.8.6 TR1 radioimmunoassay

Antibodies, tracer, standards, controls and samples were all warmed to room temperature prior to setting up of the assay. All samples, standards and controls were assayed in duplicate. For the assay 100 µl of ^{125}I-TR tracer (~15,000 cpm) was pipetted together with 100 µl standard, control or sample. Primary anti-TR1 antibody (100 µl; final dilution 1: 30,000) was then added to all tubes (with the exception of the total counts) which were vortexed and incubated at 4°C overnight.

The following day, pre-precipitated second antibody (donkey anti-rabbit serum) prepared as described above (section 2.2.8.2) was added to each tube (except the total counts), vortexed, and incubated at room temperature with shaking for 1 hour.

Following this, 1.5 ml wash solution (0.05 % v/v Brij 35 and 0.001 % w/v microcrystalline cellulose) was added (except to the total counts tubes) and the tubes centrifuged at 3000 *x g* for 30 min at 4°C. The supernatant was decanted and the precipitate was washed with a further 1.5 ml of wash solution. ^{125}I radioactivity in the precipitate was counted in a multi-well 1261 MULTIGAMMA Gamma Counter (Wallac, Gaithersburg, MD, USA). The standard curve was plotted and results interpolated using a MultiCalc data processsing package (LKB 1224-RIACalc RIA evaluation program) (Wallac, Gaithersburg, MD, USA).

Results were corrected for protein measured by the Bradford method (section 2.2.5). Intra-assay and inter-assay precision data as previously calculated by Dr Michelle Lewin (Lewin, 2003) using 9 replicates of each pool (5 and 25 µg TR/ l) were read from a single standard curve. The intra-assay CVs (%) were 14.4 and 3.9 for 5 and 25 µg TR/ l respectively. The inter-assay CV (%) was determined by running the controls in each assay for 12 consecutive assays. They were 8 and 11.1 for 5 and 25 µg TR/ l respectively.

The minimum detection limit of the TR RIA was 1.50 µg TR/l. The working concentration range of this assay was 3.50 - 50.0 µg TR/l and was defined as the concentration range which had a CV of less than 10 %. These data were calculated by Dr Lewin (Lewin, 2003) from 9 assays.

2.2.9 Thioredoxin reductase 2

The assay was performed as for TR1 (see section 2.2.8).
Standards were prepared by diluting a stock solution of purified human TR2 (1 mg TR/l) with FBS to give the following concentrations: 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 µg TR/l. Two controls were made by diluting a stock solution of human placental cytosol with FBS to give values of approximately 9 and 30 µg TR/l. Both standards and controls were dispensed into aliquots and frozen at - 80°C.

2.2.10 Taqman Real-Time PCR

2.2.10.1 Primers and probe concentrations

The relative expression of α-1 pro-collagen mRNA was assessed using Quantitative Taqman Real-Time PCR. The primers and probes used were of the following concentrations:

The forward primer concentration was 13 pmole/µl,

The reverse primer concentration was 22 pmole/µl and

The probe concentration was 36 pmole/µl.

These stocks were diluted using RNA-free water into intermediate stocks from where it can be diluted 25 times in the reaction mix of the taq-man plate. Final concentrations were 250 nM/250 nM/100 nM (For/Rev/Probe).

2.2.10.2 RNA extraction and mRNA measurement

To extract the RNA of harvested cells, the cells were collected and resuspended in lysis buffer (RLT buffer, Qiagen,West Sussex, UK). Then the RNA was extracted with the RNeasy mini-kit (Qiagen, Qiagen,West Sussex, UK) following the manufacturer's protocol. The quantity and the quality were assessed using a Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies Inc, Wilmington, DE, USA).

The Quantitative Taqman Real-Time PCR was the method used to assess the expression of α-1 pro-collagen mRNA. The protocols of the manufacturer were followed and ABI Prism 7900 Sequence Detector (Applied Biosystems) was used to assess the level of expression.

2.2.11 Oil-red O staining

This method was used to stain the fat droplets accumulated in the cells. The method used was previously described by Green and Kehinde (1975). The cells were stained kindly by Dr Forbes Howie as follows:

0.2 g of oil red O was dissolved in 200 ml of isopropanol. The solution was warmed to 56 ^{o}C for at least 1hr and then cooled and stored at room temperature. Working solution was then prepared immediately before use by adding 4 parts of distilled water to 6 parts stock solution. The working solution was then vortexed and left for 10 min. Finally it was filtered through a fine filter paper (No. 42 Whatman).

Cells were cultured on cover slips for the purpose of staining. After completion of the experiment, cells were fixed with 10% formalin for 10 mins and washed in 60% isopropanol for 30 seconds. Oil red O staining was conducted for 10 minutes. The cells then washed in 60 % isopropanol for 5 seconds. After that, they washed in distilled water and Mayer's haematoxylin solution (1 ml) was then added for 30 secs. Washing was repeated with distilled water. Cells were then counter stained with sodium phosphate for 5 min and air-dried. Finally they were mounted using a water-based preservative.

2.2.12 Selenium measurements in blood samples

Selenium was assessed by Mr.Gordon Marr using atomic absorption spectrophotometry.

A Perkin Elmer AA800 graphite furnace with Zeeman-effect background correction (Perkin Elmer Life and Analytical Sciences, Norwalk, Connecticut, USA) was used to assess selenium concentrations in the collected EDTA plasma samples.

Samples were taken in EDTA tubes and plasma separated. Plasma samples were diluted two-fold in 0.2% HNO_3. A Pd/Mg in HNO_3 matrix modifier was used to

stabilise the selenium. The method of additions calibration was used to cancel out any matrix effects in the sample. Frozen human EDTA was spiked to produce a calibration curve.

For the selenium measurements, a palladium matrix modifier was used because of its higher pyrolysis temperature and therefore a reduction in interference. This produced a more sensitive assay for serum.

Zeeman-effect background correction

Despite using a matrix modifier and high atomisation temperatures, other elements such as phosphate and iron can still interfere with the selenium measurement.. To decrease interference from these elements the Zeeman-effect background correction system was used on the AA 800. Basically, atomic lines subjected to a strong magnetic field are split into three or more parts. The extra lines occur when the magnet splits the electron energy levels in the atoms. The three lines include one at the original wavelength and two either side, at the simplest split. The higher the magnetism the greater the split. This allows atomic species to be split from non-atomic species and the difference being noted. Using the Zeeman-effect background correction can stop interference from phosphate and iron at levels of 50mg/l

The lower detection limit for selenium in this method was 0.54 µg/l. This high sensitivity of the method was achieved by using the electrode-less discharge lamp (EDL) lamp.

Pool samples were used to indicate the precision of the method. The inter-assay and intra-assay variations were 9.8 % CV and 6.6 % CV, respectively.

The bias (accuracy) calculated by measuring known selenium concentrations from the UK Trace Element External Quality Assessment Scheme (UK TEQAS) was –10%.

2.2.13 Cytokine measurements

The concentrations of six cytokines were kindly measured by Dr Suzanne Mackenzie using the commercially available BD Cytometric Beads Array (CBA). These cytokines were interleukin-8 (IL-8), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor-α (TNFα) and interleukin-12p70 (IL-12p70). This kit basically can assess multiple analytes in a single sample and with combination of flow cytometry, the method could attain a sensitivity ranging from 1.9 pg/ml for IL-12 up to 7.2 pg/ml for IL-1β with no cross reactivity between the assessed analytes.

2.2.14 Statistical analysis and computer programmes used

One-way ANOVA with Tukey post-test (comparing each column to others) was used as the statistical test unless otherwise mentioned. Graph-Pad Prism 4.00 software (Graph-Pad Software Inc., San Diego, USA) was used to create the figures. Two-way ANOVA with Bonferroni post-tests was used in chapter five.

3 CHAPTER THREE

THE EFFECT OF SELENIUM DEPLETION AND REPLETION ON SELENOENZYME EXPRESSION OF C3A AND LX-2 CELLS

3.1 Introduction:

One of the primary hypotheses of this thesis is that selenium deficiency in hepatocytes and stellate cells leads to unopposed oxidative stress giving rise to an inflammatory response which in turn activates fibrogenesis in the stellate cells. Selenium is a trace element necessary for the expression of selenoenzymes including the family of thioredoxin reductases and glutathione peroxidases (Allan et al., 1999; McKenzie et al., 2002). Selenium is required in sufficient amount in tissues to ensure that the selenoenzymes are maximally expressed (McKenzie et al., 2002) and this promotes optimal anti-oxidant protection. When selenium availability is inadequate, sub-optimal expression of selenoenzymes occurs leaving cells vulnerable to oxidative damage (McKenzie et al., 2002). To be able to investigate the induction of selenoenzymes by selenium supplementation, there is a need to develop a culture system that is selenium-deficient but allows at the same time maintenance of adequate cell viability.

The selenium content of basal culture media vary; for example, basal DMEM for mouse hybridomas contains 1 nM of selenium, but RPMI 1640 medium used to culture human hepatoma cells contains 30 nM selenium (Freshney, 1992). The DMEM basal medium used previously in our laboratory had a very low selenium concentration of 0.35 nM (Lewin, 2001), and is classified as selenium-deficient medium. Various medium supplements also have different selenium concentrations; for example, the selenium content of FBS may vary, with values ranging from undetectable to 200 nM (Leist et al., 1996), 480 nM (Fujiwara et al., 1999), and 160-180 nM (Geiger et al., 1993). Selenium found in FBS and other protein supplements

is exclusively incorporated covalently into the proteins of the serum supplements. As such many believe that selenium in these selenoproteins is neither bioavailable nor readily accessible by cells in culture (Brigelius-Flohé et al., 1995; Marcocci et al., 1997). For this reason experiments concerning the effect of selenium supplementation on promoting the synthesis of selenoproteins in cells require selenium to be added as either sodium selenite or selenomethionine. These compounds can readily enter the cell and exert a biological action through being converted to active selenide moieties that can be used to synthesize a selenocysteine residue at the active site of selenoenzymes (Baker et al., 1998; Brigelius-Flohé et al., 1995). Sodium selenite can prove toxic in doses in excess of 1µM but at the concentration used to supplement cells (5-200 nM) no such toxicity is observed.

It is well established that cell culture is a useful tool to investigate *in-vitro* various metabolic disorders. One of the aims of this study was to produce an *in-vitro* liver cell model that comprised cells grown in a wide range of selenium environments. The concentration ranges of selenium studied should cover the range from markedly selenium deficient to levels of selenium in excess of those required to maximally express selenoenzymes without being toxic. This range would encompass the selenium status of the UK population who is regarded as having only approximately 40% of the Selenium intake required to optimise the expression of GPX in red cells (Brown et al., 2000). In order to develop these models of human liver, C3A and LX-2 cells were used to represent human hepatocytes and human hepatic stellate cells, respectively. Culture conditions that would allow these cells to remain viable in selenium-deficient medium were required and, furthermore, it was important to confirm that these cell lines would respond to selenium supplementation added as sodium selenite by up-regulating the expression of the TR and GPX selenoenzymes.

The aims of this chapter were to establish culture conditions that allowed the following:

1. Maintenance of cell viability under selenium-deficient and replete conditions for C3A and LX-2 cells.

2. Induction of thioredoxin reductase 1 and glutathione peroxidase 1 expression after selenium-supplementation.

3.2 Methods

General protocols:
For experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in section 2.2.1. The cells were passaged and re-plated into two 6-well (35 mm) or 12 well plates for each cell type. After 24 hr incubation (i.e. at confluence) treatments were started as described below.

Protocol 3.2.1: Sodium selenite was added at increasing doses, 0, 5, 10, 40, 50, 100 or 200 nM. Cells were then incubated for a further 72 hours, a time that allows a new steady state for selenoproteins expression. Following this incubation period, the cells were harvested (section 2.2.2). Thioredoxin reductase activity was measured according to the method described in section 2.2.3.1. TR1 content was measured by RIA as described in section 2.2.7.

Protocol 3.2.2: The medium was changed to the one of the following media for a further 72 hours: DMEM medium with added IT (10 µg/ml Insulin /5 µg/ml Transferrin, DMEM medium with IT (10 µg/ml insulin/5 µg/ml transferrin) plus selenium (100 nM), 10 % FBS-supplemented DMEM medium, 10 % FBS-supplemented DMEM medium plus selenium (100 nM). LDH levels in cells and media were then assessed as described earlier.

Protocol 3.2.3: The medium was changed to IT-supplemented medium, (insulin [10 µg/ml] / transferrin [5 µg/ml]). After further incubation for 24 hours, sodium selenite was added at increasing concentrations, 0, 5, 10, 50, 100, 200 nM. Cells were then incubated for a further 72 hours. Following the incubation, the cells were harvested and TR activity, TR1 content and GPX1 activity assays were conducted.

Protocol 3.2.4: The medium was changed to IT-supplemented medium. After a further 24 hours of culture, sodium selenite (100 nM) was added to the cells for 72 hours. Cells then were exposed to IT (10 µg/ml (I) /5 µg/ml (T)) supplemented medium (i.e. no selenium present). Cells were collected at 0, 24, 48, 72, 96 hours

after commencement of selenium depletion. Harvested cells were stored at - 80°C until enzyme assays were carried out for both TR1 protein content and GPX1 activity. Collection and assessment of the TR1 and GPX1 activity in these cells has been described in section 2.2.2 and 2.2.7 and 2.2.3.2 respectively.

Protocol 3.2.5: The medium was changed to IT-supplemented medium. After a further 24 hours of culture, sodium selenite (100 nM) was added to the cells for 72 hours. Following the incubation, the cells were harvested and TR2 assay was conducted as described in section 2.2.8.

3.3 Results

3.3.1 Optimization experiments

3.3.1.1 Thioredoxin reductase 1(TR1) expression and activity response of C3A and LX-2 cells to increasing selenium supplementation:

The intracellular expression of TR1 in cultured C3A cells was compared to that in cultured LX-2 cells using defined culture conditions. Experiments in this section were done according to protocol 3.2.1.

It was important to demonstrate that these cells expressed significant levels of TR1 and furthermore that the expression of TR1 is responsive to increasing doses of selenium. Culture medium supplemented with 10% FBS was chosen in the first instance to allow cell plating and viability assessment as FBS is frequently a component of many typical cell culture media. It was hoped that this 10 % FBS-supplemented medium might serve as selenium-deficient medium allowing selenoenzyme expression to increase in response to addition of selenium. If this occurred then the medium would be suitable for further studies into the effect of selenium supplementation.

As shown in figure 3.1A, C3A cells grown in 10 % FBS responded to additional selenium supplementation by increasing levels of TR1 activity. TR1 content showed a similar response (figure 3.1B) to increasing selenium supplementation suggesting that the increased thioredoxin reductase activity largely reflects changes in TR1 expression. Selenite addition achieved approximately a 2-fold increase in TR1 expression with optimal induction at 40nM selenite.

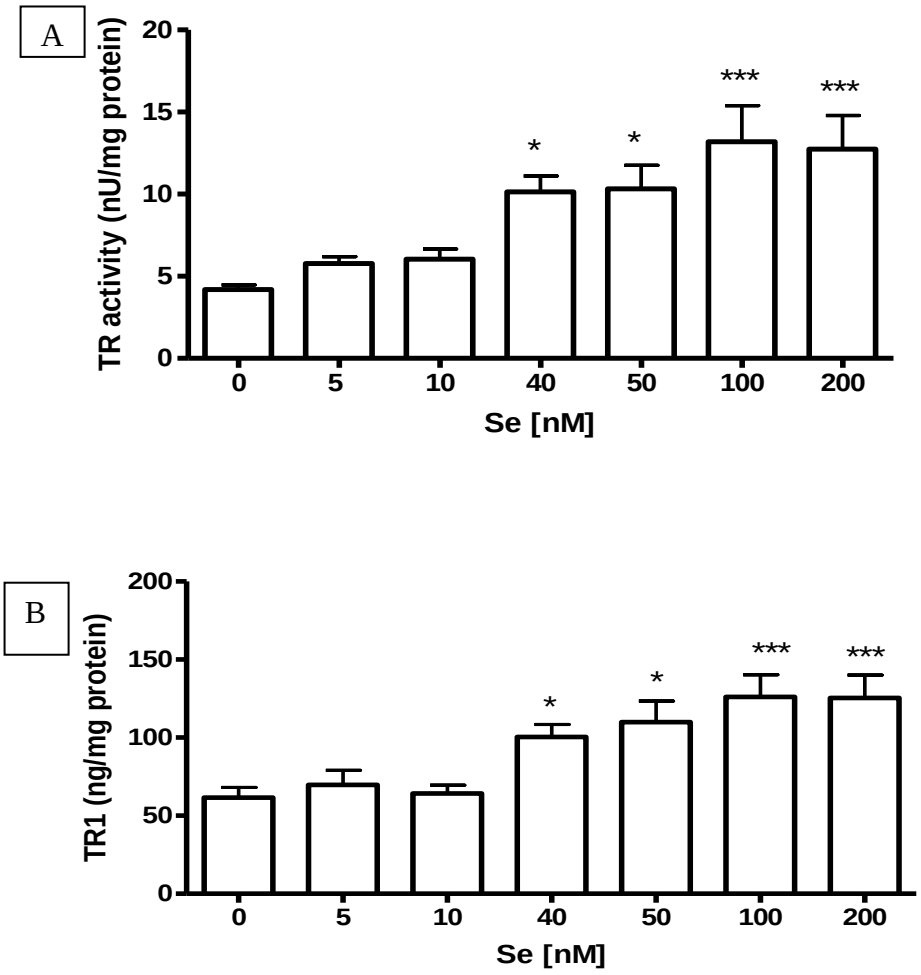


Figure 3.1: (A) TR activity and (B) TR1 protein level in C3A cells.
The response of (A) TR activity and (B) TR1 protein levels to selenium supplementation is shown. The cells were treated with increasing selenium concentrations (as sodium selenite) for 72 hours. TR activity and TR1 levels on harvested cells were assessed as described in 2.2.3.1 and 2.2.7 respectively. Measured activity represents both cytoplasmic and mitochondrial thioredoxin reductases. Data represent the mean ± standard error (± SEM). (A) n=3 and (B) n=4 experiments, each data point analysed in triplicate within each experiment. * $p<0.05$, *** $p<0.001$ compared to 0 nM Se value.

Interestingly, as shown in figure 3.2, the hepatic stellate LX-2 cells behaved differently to C3A cells. Both TR activity and TR1 content were not induced by selenium supplementation of 10% FBS culture medium, even at doses of selenium as high as 200 nM.

To investigate the possibility that LX-2 cells were not responsive because of the potential bioavailability of selenium contained in the 10% FBS culture medium, the experiment was repeated under conditions where FBS was absent from the culture medium. However, the cells failed to survive over the 72 hours incubation period of the experiment (results not shown). At this point of the study, it was necessary to identify a medium which allowed maintenance of LX-2 cell viability in the absence of FBS and selenium.

FBS-free medium is commonly supplemented with BSA to help maintain a protein balance for tissue culture. For that purpose BSA of various concentrations (0-0.75%) was used to serve as a selenium deficient medium but it also failed to maintain cell viability (see appendices A.3.2.1).

Subsequent experiments (detailed in appendices A.3.2.2) established that cell viability for LX2 cells was possible in basal medium supplemented with insulin and transferrin (IT medium) in the absence of added selenium.

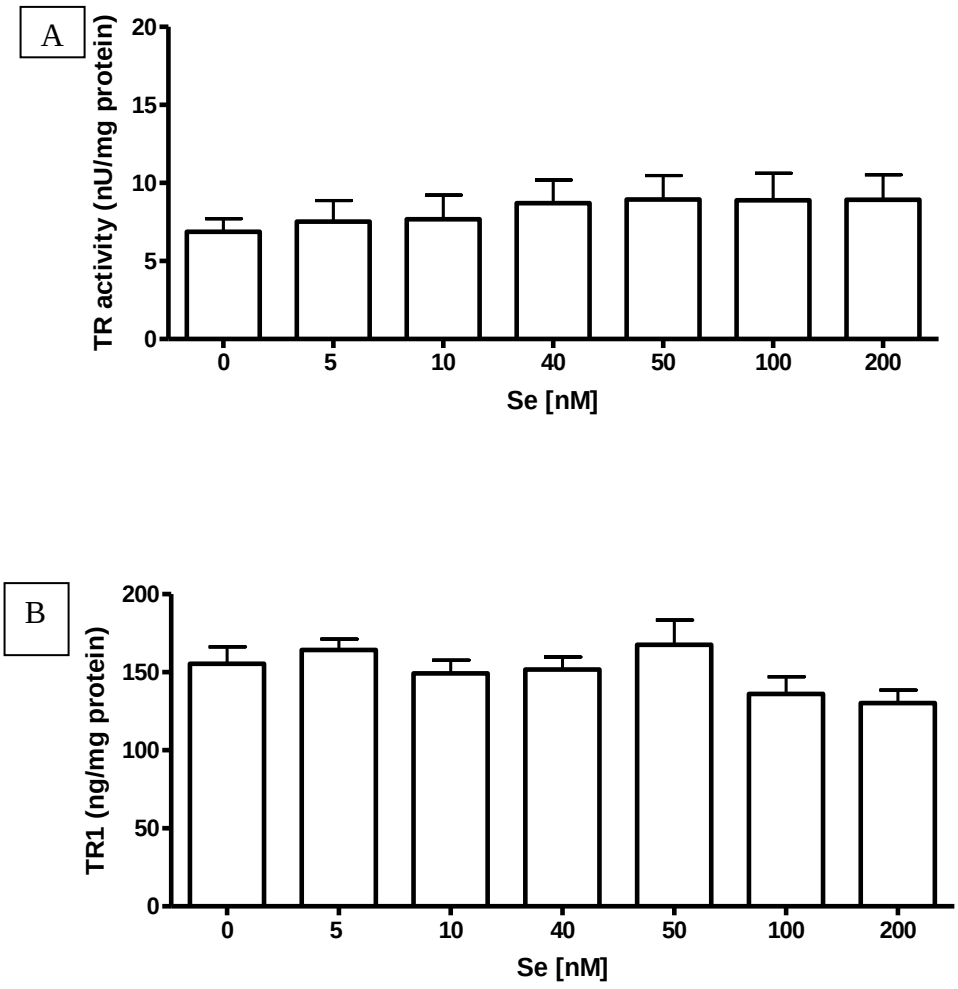


Figure 3.2: (A) TR activity and (B) TR1 protein level in LX-2 cells.
The response of (A) TR activity and (B) TR1 protein expression to selenium supplementation over the selenium range 0 to 200 nM for 72 hours. Cells then were collected and assessed at 72 hours. TR activity represents total TR, including cytoplasmic and mitochondrial TR. The graph shown represents the mean ± standard error (± SEM) for (A) n=3 and (B) n=4 experiments, each data point analysed in triplicate within each experiment.

3.3.1.2 Comparison of insulin/transferrin supplemented medium with FBS-supplemented medium on TR1 and GPX1 response to selenium supplementation of C3A and LX-2 cells:

To illustrate further the utility of IT-supplemented DMEM medium as compared to 10 % FBS supplemented medium, experiments were conducted as described in protocol 3.2.2.

Figure 3.3 confirms that selenium supplementation of IT medium significantly increases the level of TR1 in C3A and LX-2 cells compared to that in cells treated with selenium-deficient IT medium. TR1 content is at a similar level to cells grown in FBS-supplemented medium. Furthermore, as before, LX2 cells grown in FBS-supplemented medium were unable to show TR1 responsiveness to added selenium

Glutathione peroxidase 1, like TR1, is a major selenoenzyme that is thought to contribute to cell protection from oxidative damage. Glutathione peroxidase 1 is the isoenzyme found in cytosol and thus protects the same cellular compartment from oxidative damage as TR1. Moreover, glutathione peroxidase 1, like TR1, responds to selenium supplementation (McKenzie et al., 2002).

As shown in figure 3.4, GPX1 activity measured in both C3A and LX-2 cells cultured in the IT-supplemented medium plus or minus selenium was responsive to selenium supplementation.

LX-2 cells grown in FBS had significantly ($p<0.001$) higher GPX1 activity than cells grown in IT, again suggesting that a selenium source in FBS was available to the cells to promote selenoenzyme synthesis (figure 3.4B). In C3A cells selenite (100nM) stimulated GPX1 activity in cells grown in IT media to the same level as that seen in FBS treated cells exposed to selenite (figure 3.4A).

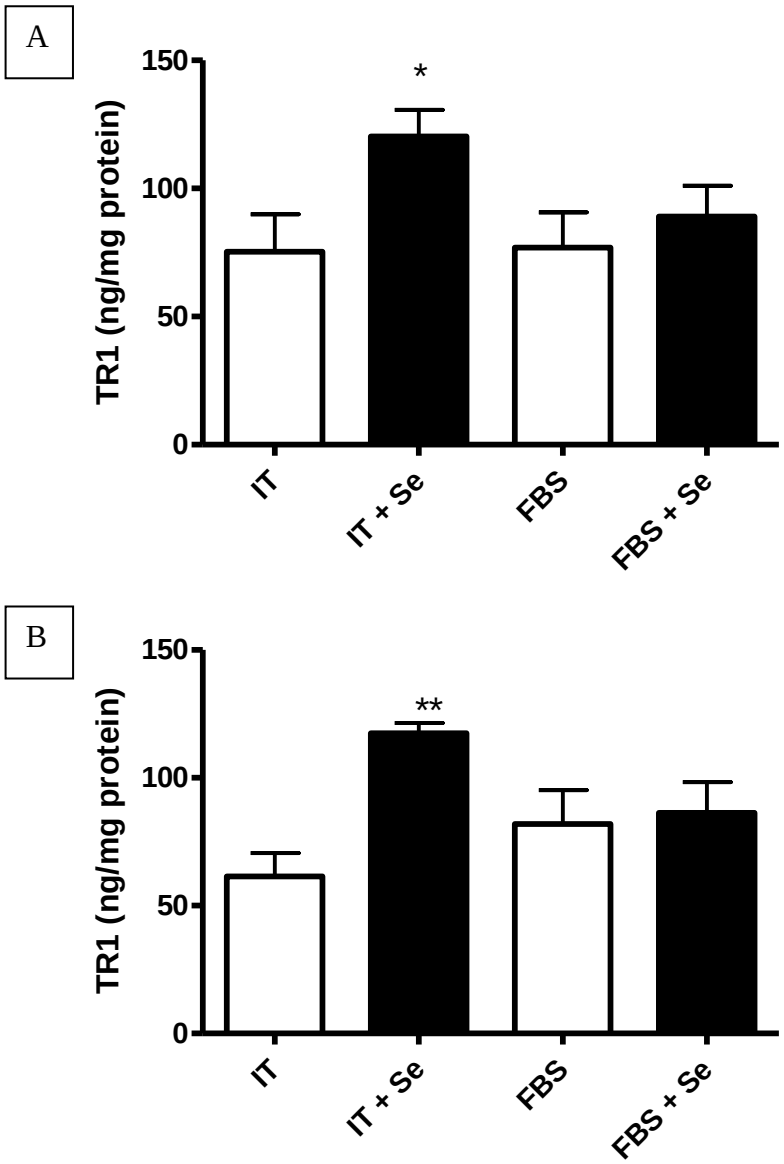


Figure 3.3: Effect on selenium supplementation of various media on TR1 protein level of (A) C3A and (B) LX-2 cells.

Both C3A and LX-2 were plated in DMEM medium containing 10 % FBS. After 24 hours, the medium was removed and the different supplements as shown were added for 72 hours. Cells were then collected and assessed for TR1 levels. The selenium concentration was 100 nM. These experiments were done in triplicate in three different experiments. The graph shown represents the mean ± standard error (±SEM). (n=3), * $p<0.05$, ** $p<0.01$ relative to IT (control).

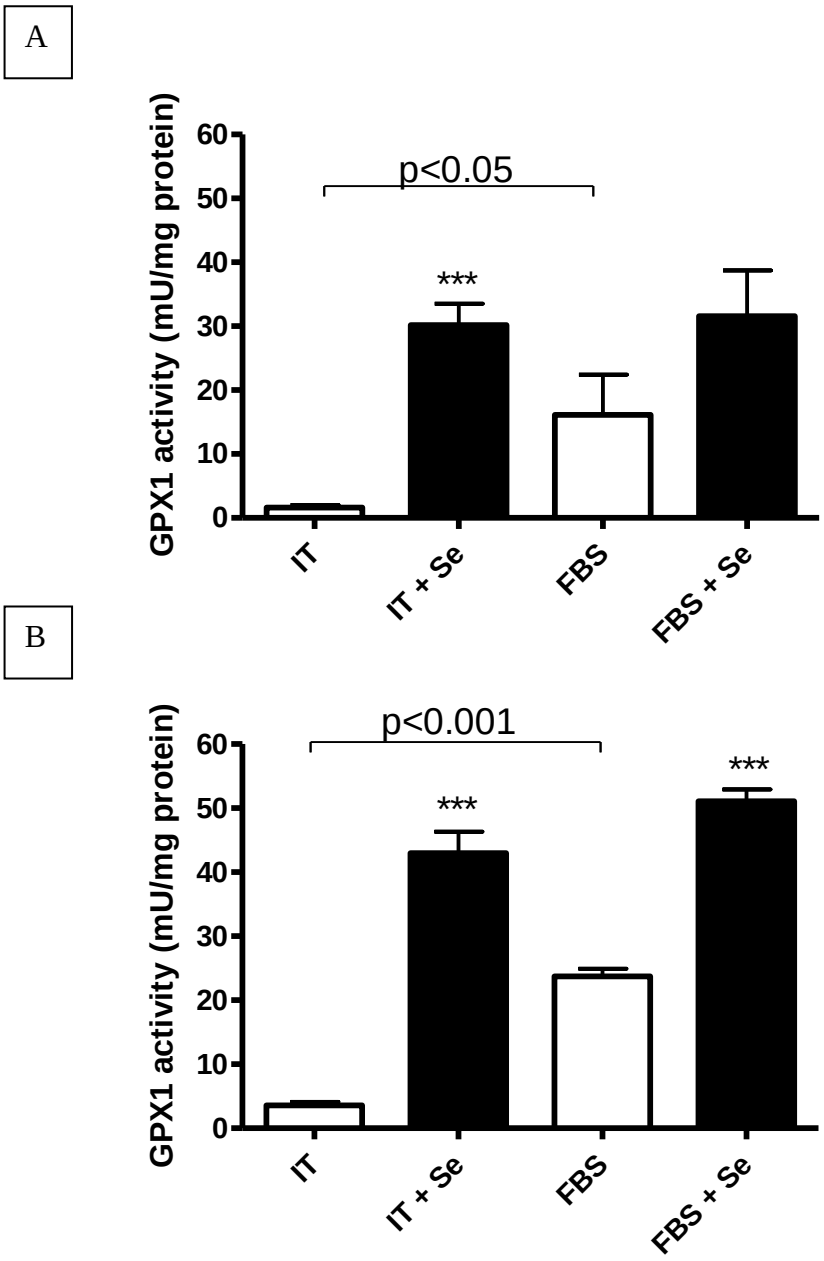


Figure 3.4: Glutathione peroxidase 1 activity in (A) C3A and (B) LX-2 cells after selenium (100nM) supplementation.

Both (A) C3A and (B) LX-2 were plated in DMEM medium containing 10 % FBS. After 24 hours, the medium was changed to IT-supplemented medium or 10 % FBS-supplemented medium for 24 hours. 24 hours later, sodium selenite (100 nM) was added for a further 72 hours. The graph shown represents the mean ± standard error (±SEM) of 3 experiments. Each experiment was done using triplicate wells for each data point. *** $p<0.001$ relative to respective IT or FBS controls.

3.3.2 Hypothesis driven experiments

3.3.2.1 Dose response of selenium supplementation on TR1 content and GPX1 activity of C3A and LX-2 cells:

The purpose of this experiment was to confirm that cells grown in IT-supplemented medium were responsive to increasing doses of selenium and to determine levels of selenium supplementation that allowed maximal expression of TR and GPX (see protocol 3.2.3).

As seen from figure 3.5 and 3.6, both TR1 and GPX1 increased in response to increasing amounts of selenium supplements. Optimal expression of TR1 and GPX1 was achieved at a selenite dose of 50 –100nM. While TR1 increased 2-fold, GPX1 showed a 10-fold increase at the optimal dose of selenite (50-100 nM).

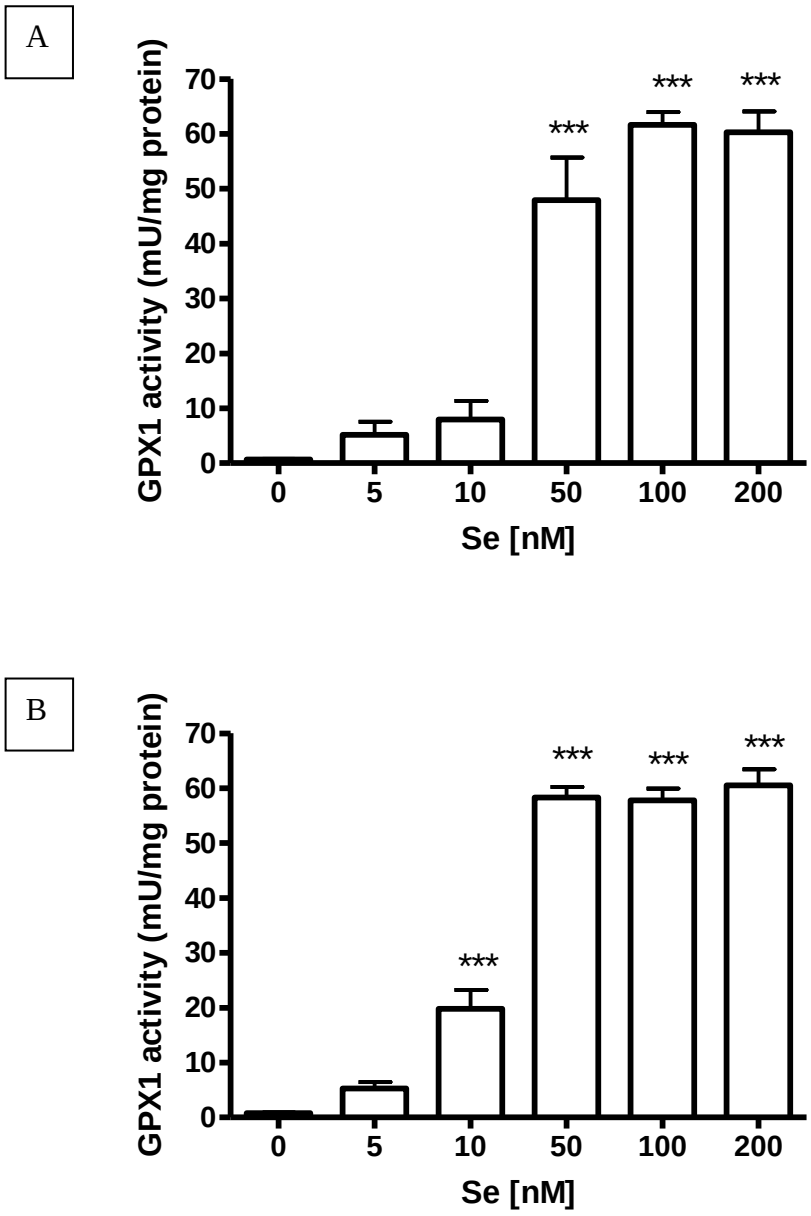


Figure 3.5: Glutathione peroxidase 1 activity response to selenium supplementation when grown in IT-supplemented medium in (A) C3A and (B) LX-2.
Both C3A cells and LX-2 cells were plated and grown in DMEM medium containing 10 % FBS. After 24 hours cells were placed in IT-supplemented medium for 24 hours. After a further 24 hours, selenium at the concentrations shown was then added for a further 72 hours. This graph represents mean ± standard error (±SEM) for 3 different experiments. Each data point was analysed using triplicate wells within each experiment. (***)= $p<0.001$ relative to 0 nM Se condition

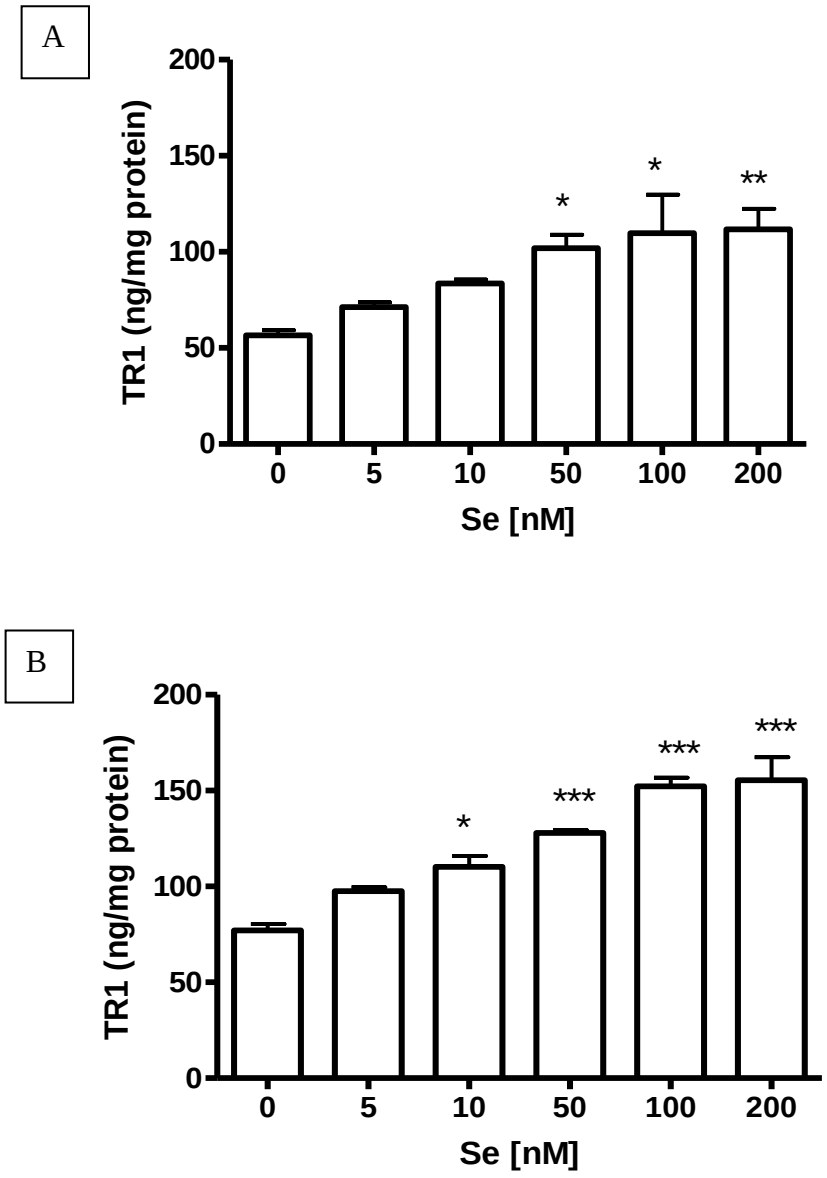


Figure 3.6: Response of TR1 protein levels to selenium supplementation (IT-supplemented medium) in (A) C3A and (B) LX-2 cells. Both C3A cells and LX-2 cells were plated and grown in DMEM medium containing 10 % FBS. After 24 hours cells were placed in IT-supplemented medium for a further 24 hours. Selenium at the concentrations shown was then added for a further 72 hours. Cells were then harvested and TR1 content assessed. This graph represents mean ± standard error (±SEM) of three independent experiments where each data point within an experiment was analysed using triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to 0 nM Se condition.

3.3.2.2 Hierarchy study of TR1 and GPX1 profile in C3A and LX-2 cells in selenium-deficient medium:

To assess the hierarchy of retained selenoprotein expression in selenium deficiency experiments were conducted according to protocol 3.2.4.

Figure 3.7 A illustrates that GPX1 activity was quickly lost from C3A and LX-2 cells when cells that had been exposed to optimal doses of selenite were switched to selenium-deficient medium. After 72 hours in selenium deficient media, the GPX activity remaining in C3A and LX-2 cells was only 25% and 10% respectively of that found at time zero (figure 3.7A). In contrast, TR1 showed only a modest fall in content over this time period decreasing to 63% of content in C3A cells and 71% of the content in LX2 cells (figure 3.7B).

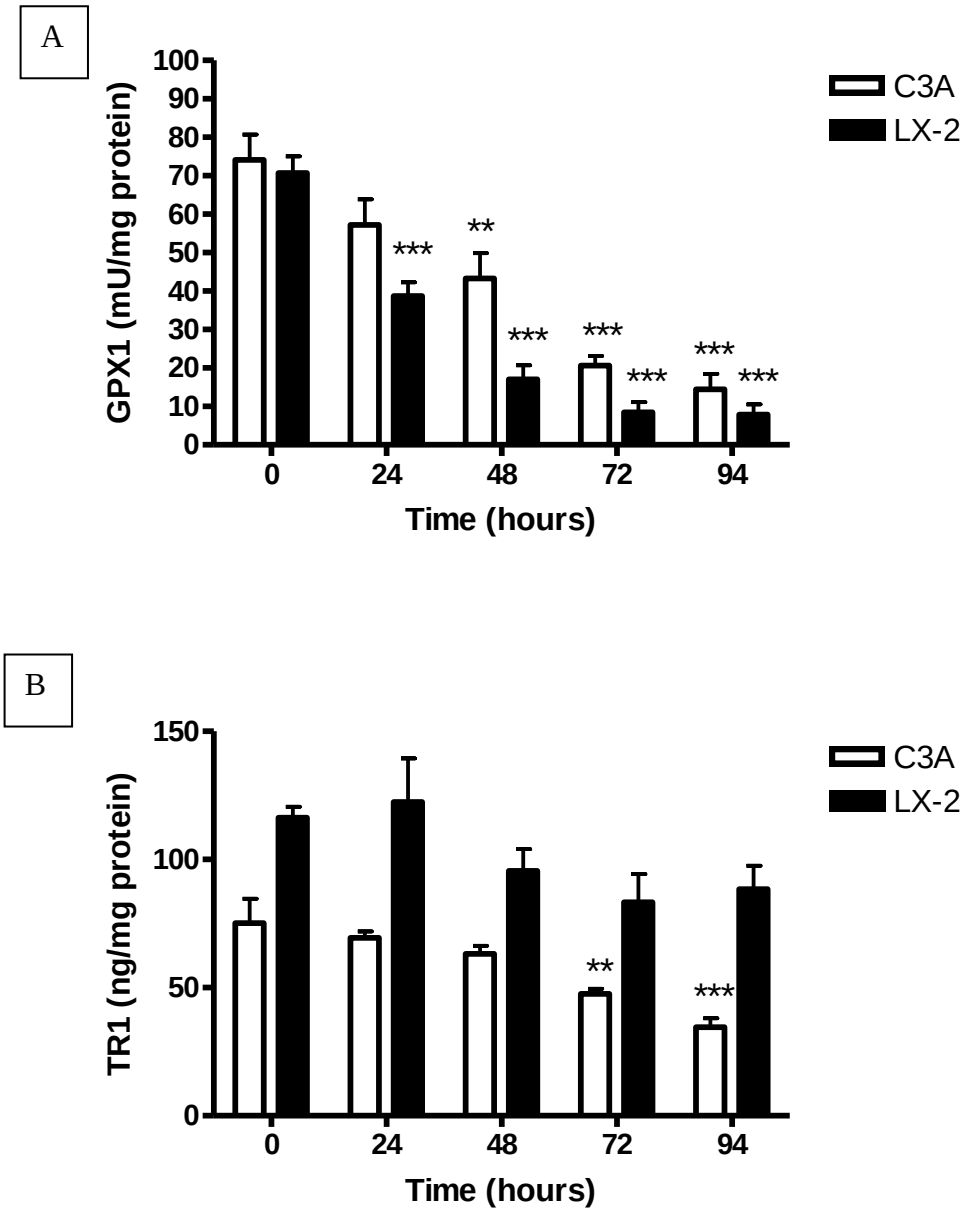


Figure 3.7: The effect of selenium depletion on (A) GPX1 activity and (B) TR1 content of C3A and LX-2 cells:

Both (A) glutathione peroxidase 1 activities and (B) thioredoxin reductase 1 protein levels were assessed. C3A and LX-2 cells were plated in DMEM medium containing 10 % FBS. After 24 hours, the medium was changed to IT-supplemented medium. Selenium (100 nM) was then added for a further 72 hours. Cells then were exposed to selenium-deficient IT (10 µg/ml (I) /5 µg/ml (T)) supplemented medium. Cells were collected at 0, 24, 48, 72, 96 hours after commencement of selenium depletion and the GPX activity and TR1 protein expression measured. The data represent mean ± standard error (±SEM) of three independent experiments with each data point analysed in triplicate within each experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ relative to the respective zero time control.

3.3.2.3 The level of thioredoxin reductase 2 is not affected by selenium status:

Thioredoxin reductase 2 is an important selenoenzyme that is expressed mainly in the mitochondrial compartment of the cell. The experiment in this section was done according to protocol 3.2.5.

Figure 3.8 illustrates that thioredoxin reducatse2 did not respond to added selenium in either C3A or LX-2 cells.

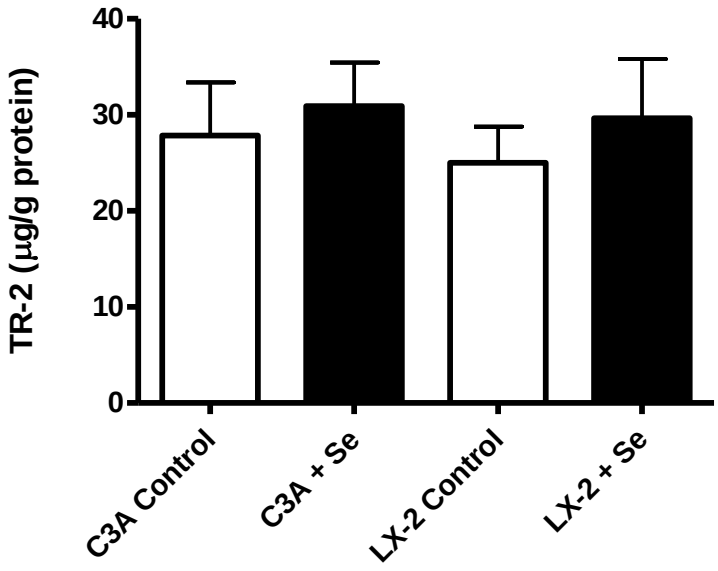


Figure 3.8: The response of TR2 to addition of an optimal selenium concentration [100 nM]: The medium was changed to IT-supplemented medium after 24 hours of plating. After a further 24 hours of culture, sodium selenite (100 nM) was added to the cells for 72 hours. Following the incubation, the cells were harvested and TR2 assay was conducted as described in section 2.2.8. The data represent mean ± standard error (±SEM) of three independent experiments with each data point analysed in triplicate within each experiment.

3.4 Conclusion and Discussion:

Conclusions:

1. IT was identified as an appropriate supplement to be used for future studies in this thesis, meeting all the criteria mentioned at the beginning of this chapter (i.e. allowing good cell viability in a medium that is deficient in selenium and alleviating a requirement for FBS).
2. Both C3A and LX2 cells demonstrate increased expression of GPX and TR1 in IT medium supplemented with selenium. The selenium dependency is dose-dependent, reaching maximal expression by 50 – 100 nM Se.
3. Subsequent selenium depletion, following maximal expression of GPX and TR1 in selenium-containing IT, demonstrated that GPX activity falls more sharply than TR1 activity, consistent with the hypothesis of a hierarchy of selenoenzyme retention under conditions of selenium restriction.
4. TR2 activity is unaffected by selenium status.

Discussion:

The results in this chapter establish that both C3A and LX-2 cells are capable of expressing similar levels of GPX1 and TR1.When C3A and LX-2 cells were grown and plated in 10% FBS-supplemented medium, LX-2 cells appeared to have access to selenium (most likely present in FBS covalently bound) and were thus able to maximally express both TR1 and GPX1. There are two possible explanations for this high expression of TR1. Firstly, higher levels of TR1 are associated with oxidative stress (McKenzie et al., 2002; Moon et al., 2005; Rundlof et al., 2007) and this might apply to LX-2 cells as these cells are partially activated cells and therefore also could be considered as partially injured or undergoing oxidative stress (Xu et al., 2005). Secondly, TR1 may be maximally expressed in LX-2 cells if these cells are able to access an adequate supply of selenium present in FBS. This latter explanation is supported by the low TR1 content when cells were grown in selenium-deficient medium (Allan et al., 1999). Furthermore, TR1 was unable to be further induced in LX-2 cells in FBS-supplemented medium containing additional selenium as sodium

selenite. The results support the hypothesis that LX-2 cells may have access to sufficient selenium in the FBS-supplement to allow maximal expression of TR1.

In contrast to LX-2, C3A cells showed a good response to selenium supplementation of FBS-supplemented medium with sodium selenite. Although, several studies have been able to induce selenoenzymes when selenite is added to 10 % FBS-supplemented medium in other cell-types (Campbell et al., 2007; Miller et al., 2002) FBS-supplemented medium was not used in any of our subsequent experiments for the following reasons:

a) The observation in this chapter indicated that LX-2 cells were capable of accessing the selenium supply incorporated into FBS whilst C3A were unable to achieve this (figure 3.2 vs 3.1). Thus it was necessary to culture both cell types in a culture medium that allowed controlled access of selenium supply to each cell type.

b) There was a clear evidence that cell viability, as assessed by the percentage LDH retention was achieved when the cells were cultured in IT medium.

Further studies were conducted to confirm the practicality of IT-supplemented medium in achieving selenium depletion of cells to allow a reproducible demonstration of selenium-dependent increases in selenoenzyme expression. The results reported in this chapter confirmed that IT medium is able to meet these requirements. Moreover, the selenoenzymes GPX1 showed lower levels of basal expression in IT-supplemented medium compared to FBS-supplemented medium and could be induced by selenium supplementation. Thus, IT-supplemented medium is a suitable choice to maintain cell viability when serum-free conditions are to be used and provides an environment where selenoenzyme expression can be induced by selenium supplementation.

TR1 showed the same level of protein expression in IT medium supplemented with selenium compared to FBS-supplemented medium. Furthermore, it was clear from experiments shown in figures 3.5 and 3.6 that the induction of GPX by addition of

selenium to selenium-deficient medium was more marked than that of TR1. This is explicable through the "Hierarchy hypothesis" concerning selenoenzymes. This hypothesis states that, under conditions of selenium deficiency, the expression of those selenoenzymes with more critical biological action is retained at the expense of less biologically important selenoenzymes. Thus, in selenium deficiency, TR1 expression is preferentially retained whilst GPX activity is lost earlier. Figure 3.7 shows that whilst GPX1 activity in LX-2 cells decreases comparatively quickly over time, TR1 largely maintains its expression over the same time course. According to the hypothesis, therefore, this difference reflects a greater hierarchical importance in diverting selenium to TR1, as opposed to GPX1 in LX-2. This observation is confirmed by several reports in other situations of selenium depletion (McKenzie et al., 2002). Although, both enzymes were decreased over time in C3A cells, GPX1 was again depleted faster than TR1 in the same cells. Moreover, in selenium-replete conditions the GPX1 response was more profound than TR1 in C3A and LX-2 cells. This might suggest that GPX1 is more important anti-oxidant in cases of selenium-replete tissue. Since the UK population as a whole has suboptimal expression of selenoproteins because of the suboptimal intake of selenium (Brown et al., 2000), TR1 may be more important than GPX1 as an antioxidant when selenium availability is limited by dietary supply, as is reported for the UK.

Several studies indicate that the mitochondrial thioredoxin reductase (TR2) knockout mouse is embryologically lethal and hence it is assumed that the TR2 selenoprotein is at the top of the hierarchy of selenoproteins when selenium supply is deficient or limited (Nonn et al., 2003). This would be consistent with the essential role that TR2 is known to have in mitochondrial function. In my experiments TR2 was not responsive to selenium depletion or repletion states in either C3A or LX-2 cells (figure 3.8). A similar maintenance of TR2 in selenium-deficient human vascular endothelial cells has been observed in our laboratory (Dr Beckett personal communication). Thus these data support the essential role of TR2 in the mitochondria of C3A and LX-2 cells, though TR2 was not subjected to further enquiry in my thesis.

4 CHAPTER FOUR

CONDITIONS FOR FAT LOADING C3A AND LX-2 CELLS

4.1 Introduction

The pathogenesis of non alcoholic steatohepatitis (NASH) is commonly explained by the two-hit hypothesis (Day and James, 1998), where insulin resistance with fat accumulation in the hepatocyte represents the first hit. However, with prolonged exposure to fatty acid flux, the liver undergoes marked oxidative stress and it is this that is regarded as the second hit.

Development of an in-vitro model of NAFLD has been challenging since the model of the disorder, as described by the two-hit hypothesis, requires not only fat-loaded cells (and/or displaying insulin resistance) in culture but also cells that are undergoing oxidative stress.

In this chapter, LPON medium, a combination of Lactate [20 mM], Pyruvate [2 mM], Octanoate [4 mM] and NH_4Cl [4 mM] was chosen as one culture medium to fat load C3A and LX-2 cells. LPON-treated C3A cells accumulate fat vacuoles which can be observed by electron microscopy (Filippi et al., 2004). The primary rational for using the various agents in LPON is as follows: Lactate/Pyruvate promotes lactate elimination, Octanoate enhances β-oxidation, and NH_4Cl promotes ammonia elimination (Filippi et al., 2004). LPON proved to be a good model to induce insulin resistance and triglyceride accumulation (Celine Filippi, personal communications). This suggests that LPON could be used as a basis from which to develop a NAFLD in-vitro model since it leads to fat accumulation and produces cells that should be under oxidative stress due to β–oxidation of octanoate.

Other studies have examined the ability of cultured hepatocytes (Hep G2 cells) to be fat-loaded with the common dietary long chain fatty acids, either oleate or palmitate or both combined, analysing their effect on fat accumulation and apoptosis. These studies concluded that palmitate predisposes to more acute injury to the liver cells, while oleate appeared to promote a more chronic type of injury (Gomez-Lechon et al., 2007; Wei et al., 2006). Thus a second model of fat loading was studied in this chapter, involving oleate and/or palmitate

Although, NAFLD is a complicated disease where many factors are involved, this chapter will explore the development of an in-vitro model of NAFLD that exhibits triglyceride accumulation and more importantly, insulin resistance and/or oxidative stress leading to cellular damage.

Hepatic stellate cells in the quiescent state contain fat droplets, mainly composed of vitamin A. These fat droplets release their vitamin A in the activation process. The ability of these cells to release vitamin A has been tested in one study using conditioned medium from macrophages (Friedman et al., 1993) which concluded that conditioned medium contains some activation factors which contribute to the depletion of fat droplets in these stellate cells. No other studies since then have explored the activation process of these stellate cells and its relationship to vitamin A release.

The aims of this chapter were to assess fat-loaded hepatocyte and hepatic stellate cell models in terms of:

1. The degree of triglyceride accumulation.
2. Selenoenzyme expression in response to fat-loading which might reflect exposure of the cell to oxidative stress.

4.2 Methods

General protocols

For the purpose of experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in sections 2.2.1. The C3A and LX-2 cells were passaged and re-plated into 6-well plates at densities of 100 and 50 x 10^3 cells per well, respectively. After 24 hr incubation (i.e. at confluence) treatments were started as described below. Protein concentrations were measured as described in section 2.2.5. The triglyceride content was assessed as described in section 2.2.6.

Protocol 4.2.1: The cells were washed twice with HBSS and then treated with Lactate [20 mM], Pyruvate [1 mM], Octanoate [4 mM], NH_4CL [4 mM] or LPON for 48 hours. The final pyruvate concentration was 2 mM because DMEM medium already contained 1 mM sodium pyruvate.

Protocol 4.2.2: The cells were cultured in IT-supplemented medium after 24 hours of plating. After 24 hours, cells were washed twice with HBSS before treatment with oleate at [0.25 mM] or [0.5 mM] or palmitate at [0.125 mM] or [0.25 mM] contained in IT-supplemented medium for 24 hours.

Protocol 4.2.3: TR1 concentrations were assessed in LPON-loaded versus oleate or palmitate-loaded C3A and LX-2 cells as described in protocols 4.2.1 and 4.2.2 respectively. The intracellular TR1 levels of C3A cells were compared to those of LX-2 cells as described in section 2.2.8.

Protocol 4.2.4: The cells were washed twice with HBSS and then treated with lactate [20 mM], or pyruvate [1 mM], or octanoate [4 mM] or NH_4CL [4 mM] or LPON for 48 hours. TR1 and GPX1 levels were measured as described in sections 2.2.8 and section 2.2.3.2 respectively.

4.3 Results

4.3.1 Hypothesis driven experiments

Two models of fat loading C3A and LX-2 cells were explored, namely the LPON and oleate/palmitate models.

4.3.1.1 LPON versus oleate/palmitate as a fat loading model:

The intracellular triglyceride accumulation in C3A cells was compared to that in LX-2 cells using two different models to fat load them.

For LPON-treated cells, the incubation time (48 hours) was chosen based upon preliminary experiments showing that 48 hours incubation was optimal to allow triglyceride accumulation without affecting cell viability (data not shown). LPON-treated cells were treated as described in protocol 4.2.1.

Oleate and palmitate were chosen because of their common use as dietary fatty acids. Preliminary experiments were done to determine the optimal concentration (see appendix (A4.1.1) and incubation period (see appendix A4.1.2) of oleate and palmitate. Oleate- and palmitate-treated cells were treated as described in protocol 4.2.2.

As shown in figure 4.1, the triglyceride level increased in LPON-treated C3A and LX-2 cells. While C3A cell triglyceride levels increased by 50 %, LX-2 cells showed an approximately 200% increase in triglyceride content. The triglyceride level of both C3A and LX-2 cells increased with oleate or palmitate supplementation over 24 hours. Interestingly, the triglyceride level of palmitate-loaded cells [0.125 mM] was significantly ($p < 0.001$) lower than that of oleate-treated cells [0.25 mM] in both cell types.

Although, cells treated with 0.5 mM oleate or palmitate were able to accumulate more triglyceride than cells treated with 0.25 mM fatty acid (Fig 4.1), cells showed

compromised viability at [0.5 mM] of oleate or palmitate. Since it was necessary to maintain a balance of good viability versus fat-loading for these cells it was decided to use 0.25 mM oleate and 0.125 mM palmitate in subsequent experiments.

Figure 4.2 shows triglyceride accumulation using the oil red O method where red pigment concentrates in the fat droplets.

Further experiments were conducted to show the effect of combined treatments of oleate and palmitate. The results indicated that, when various combinations of fatty acids were used, triglyceride accumulation was similar to that shown in fig 4.1 (see appendix A4.1.3).

Further preliminary experiments had shown that C3A and LX-2 cells accumulated similar levels of triglyceride after LPON treatment when incubated in IT-supplemented medium in contrast to 10 %FBS-supplemented medium i.e. in this experiment (see appendix A4.1.4).

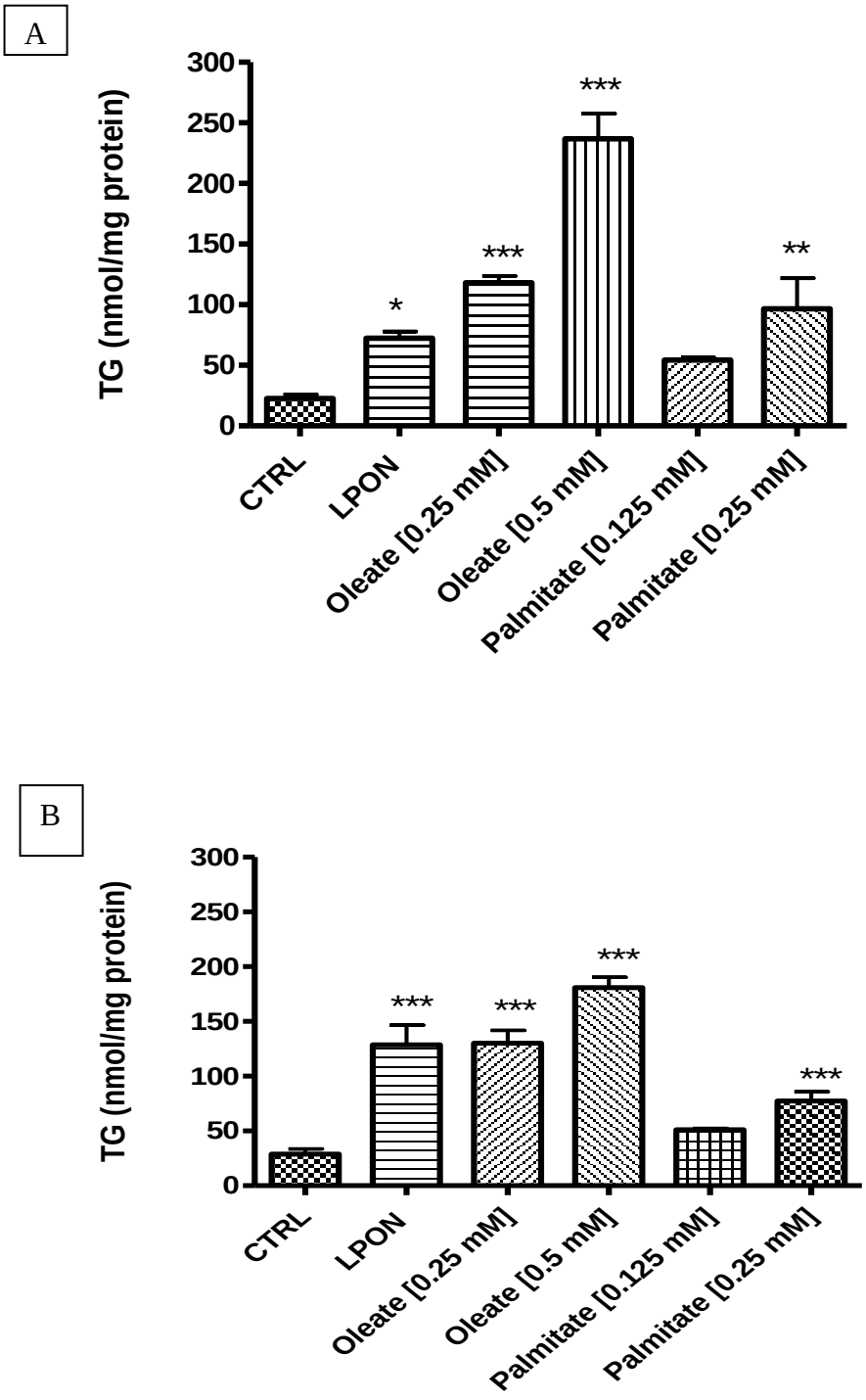


Figure 4.1: Triglyceride accumulation in LPON, oleate or palmitate-treated (A) C3A and (B) LX-2 cells C3A and LX-2 cells were incubated for 48 hours in cells treated with LPON and 24 hours in cells treated with oleate or palmitate at the concentrations shown. Triglyceride content was measured as described in section 2.2.6. Data are the mean ± standard error (± SEM) n=3 for both A and B, each experiment done in triplicate. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control which comprised no LPON or fatty acid additions.

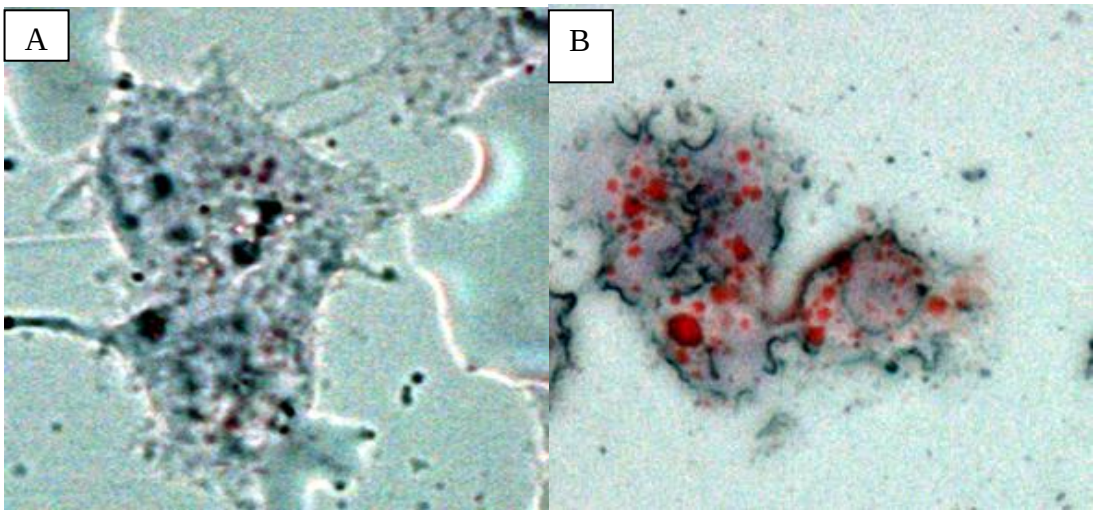


Figure 4.2: Fat accumulation in LPON-treated C3A cells
Oil red O staining of C3A cells treated with LPON for 48 hours was accomplished according to the method described in section 2.2.11. Red dots in panel (B) represent fat accumulated in LPON-treated cells, versus control cells (panel A) at the 48 hours treatment.

4.3.1.2 The effect of LPON, oleate or palmitate treatment on the expression of thioredoxin reductase1 (TR1)

The effects of LPON, oleate or palmitate on selenoenzyme expression were investigated as described in protocol 4.2.3.

As shown in figure 4.3, LPON-loaded C3A and LX-2 cells demonstrated increased expression of TR1 concentrations. Clearly, one or more of the LPON components may have been responsible for the induction of TR1 (see next section). TR1 levels were not increased in C3A and LX-2 cells by treatment with oleate or palmitate.

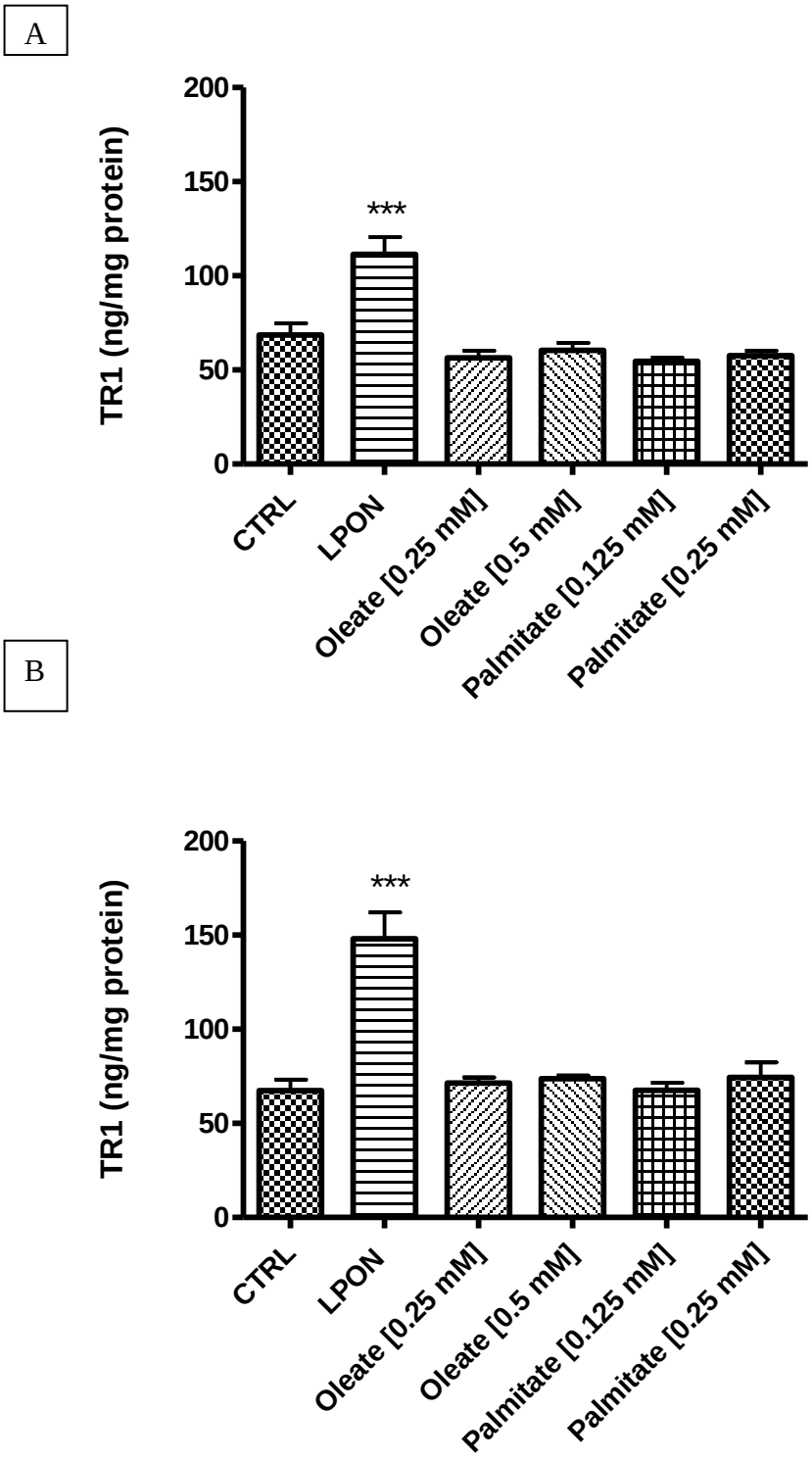


Figure 4.3: The effect of LPON, oleate or palmitate treatments on TR1 levels in (A) C3A and (B) LX-2 cells. C3A and LX-2 cells were incubated for 48 hours with LPON and for 24 hours with either oleate or palmitate at the indicated concentrations. Cells were then collected and TR1 levels were assessed as described in section 2.2.8. Data represent the mean ± standard error (± SEM) for (A) n=3 and (B) n=3 experiments, each done in triplicate. *** $p<0.001$ compared to control.

4.3.1.3 The effect of individual LPON components on triglyceride, TR1 level and GPX1 activity

To assess which component of LPON was responsible for the triglyceride accumulation and TR1 induction protocol 4.2.4 was followed.

As illustrated in figures 4.4, 4.5 and 4.6 octanoate appeared to be the single component of LPON responsible for the increased triglyceride concentration, TR1 concentration and GPX1 activity in both C3A and LX-2 cells. The other components individually had little if any effect on triglyceride levels or selenoenzyme expression.

LX-2 cells showed the highest levels of triglyceride accumulation, reaching 125 ± 20 nmol/mg protein compared to C3A cells which only reached about 90 ± 10 nmol/mg protein when treated with LPON. This difference was also observed with octanoate treatment. Other components of LPON treatment showed comparable results, with little or no effect on triglyceride accumulation.

LX-2 cells showed a different response to C3A cells when TR1 levels were compared. However, it was anticipated that the control cells would have higher levels of TR1 in LX-2 cells based upon earlier experiments reported in section 3.2.1. Higher levels of TR1 were also observed in LX-2 cells treated with LPON (200% of the control) compared to C3A cells (only 150% of the control). The same trend towards higher TR1 levels in LX2 cells was also observed in octanoate treated cells. Other components of LPON appeared to have no effect on TR-1 expression in both C3A and LX-2 cells.

Although, higher levels of GPX1 activity were observed in control LX-2 cells 30 ± 2 mU/mg protein compared to C3A cells 25 ± 2 mU/mg protein, the same percentage increase (about 160 % of control cells) was obtained in response to LPON or octanoate in both C3A and LX-2 cells. Other components of LPON again did not significantly affect the expression of GPX-1 in both C3A and LX-2 cells.

Since colleagues in the hepatology research group at Edinburgh Royal Infirmary had previously shown that the complete LPON formulation induces insulin resistance (Dr Filippi, personal communication), it was decided to continue further experiments with complete LPON supplements rather than using octanoate alone.

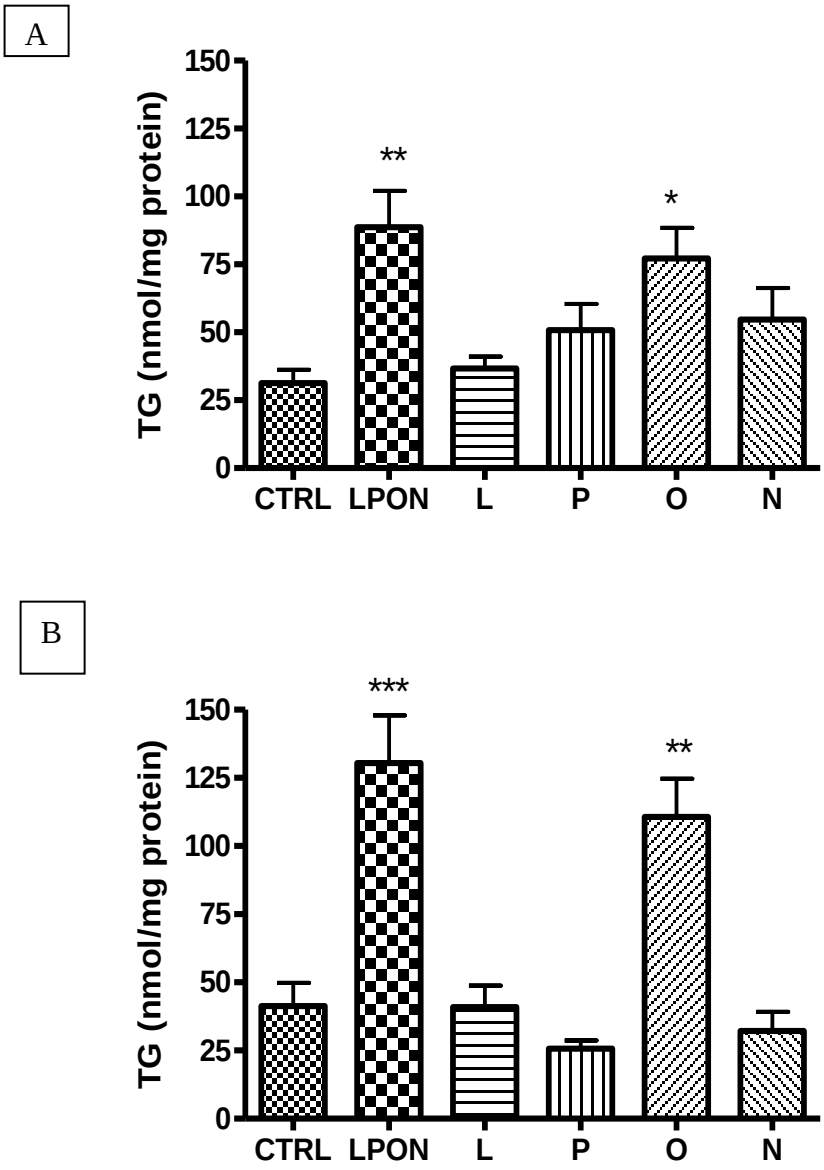


Figure 4.4: The effect of LPON and its various components on triglyceride content of (A) C3A and (B) LX-2. C3A and LX-2 cells were incubated for 48 hours with LPON or separately its various components. Cells were then collected and triglyceride content assessed as described in section 2.2.6. L=Lactate, P=Pyruvate, O=Octanoate, N= ammonium chloride (NH_4Cl). Data represent the mean ± standard error (± SEM), n=3 for both A and B, each experiment done in triplicate. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ compared to control (CTRL).

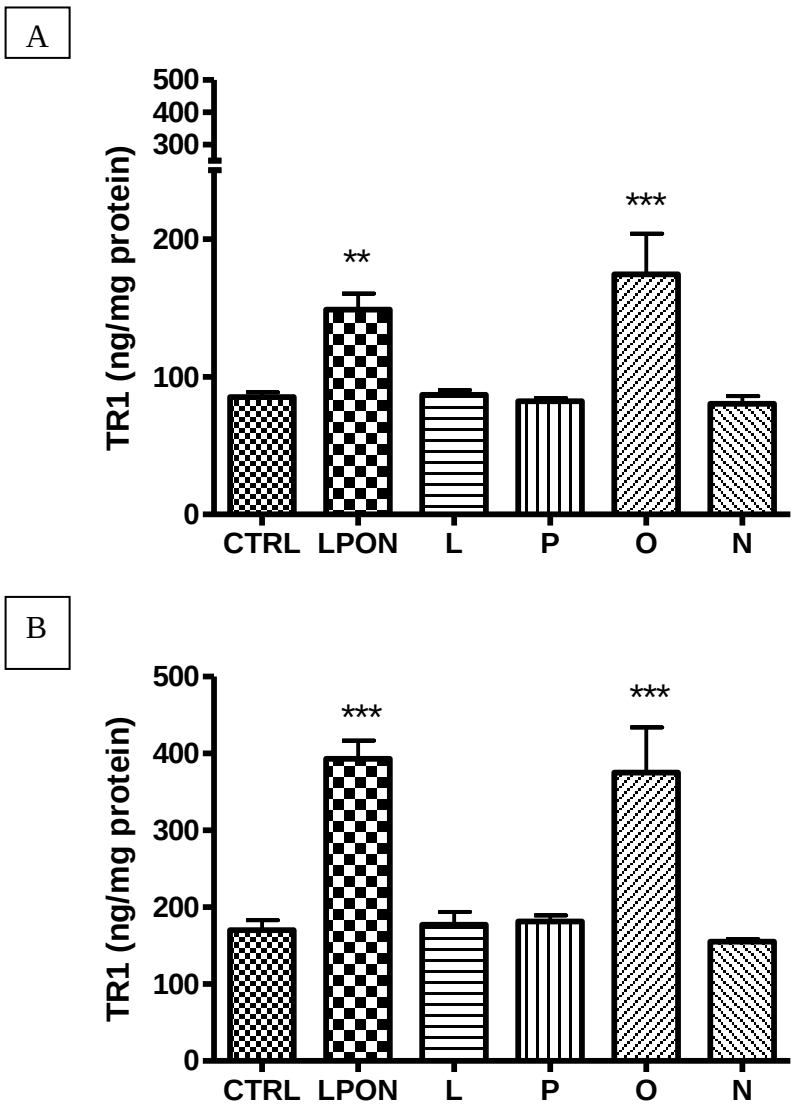


Figure 4.5: The effect of LPON and its various components on TR1 concentrations in (A) C3A and (B) LX-2. C3A and LX-2 cells were incubated for 48 hours with LPON or its various individual components. Cells were then collected and TR1 assessed as described in section 2.2.8. L=Lactate, P=Pyruvate, O=Octanoate, N= NH_4Cl). Data represent the mean ± standard error (± SEM), n=3 for both A and B, each experiment done in triplicate. ** $p<0.01$, *** $p<0.001$ compared to control (CTRL).

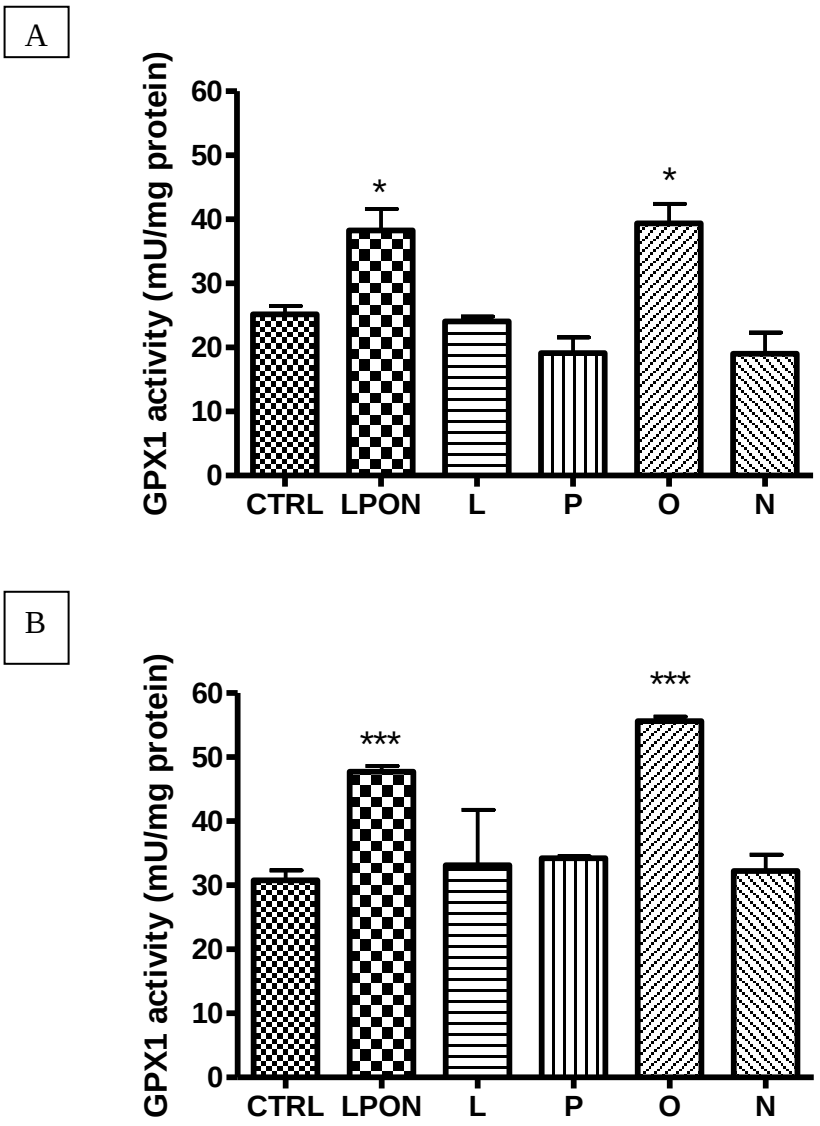


Figure 4.6: The effect of LPON and its various components on GPX1 activity of (A) C3A and (B) LX-2. C3A and LX-2 cells were incubated for 48 hours with LPON or separately its various components. Cells were then collected and GPX1 assessed as described in section 2.2.3.2. L=Lactate, P=Pyruvate, O=Octanoate, N= NH_4Cl. Data represent the mean ± standard error (± SEM), n=3 for both A and B, each experiment done in triplicate. * $p<0.05$, *** $p<0.001$ compared to controls.

4.4 Conclusion and Discussion

Conclusions:

1. Triglyceride accumulation in LX2 or C3A cells can be achieved *in vitro* by using either LPON or oleate treatment. Both treatments could thus be regarded as candidate systems for an in vitro model of NAFLD.

2. LPON-loaded cells showed increased TR1 and GPX1 expression which might imply that such cells are under a marked degree of oxidative stress. It appeared that the component responsible for this effect in LPON was octanoate suggesting that β-oxidation of the fatty acid might be responsible for the oxidative stress.

3. Oleate treatment produced the greatest degree of fat-loading but the fatty acid showed no effect on inducing TR1 or GPX1. This might imply that such cells were not under the same degree of oxidative stress as LPON treated cells. This may possibly be due to the regulated entry of long-chain fatty acids into mitochondria.

Discussion

Fatty acids are commonly classified into saturated, e.g., palmitate, (C16:0) and octanoate (C8:0), mono-unsaturated, e.g., oleate (c18:1), or poly-unsaturated fatty acids, e.g. linoleic acid (C18:2). Palmitate, linoleic acid and oleate also can be classified as long-chain fatty acids, while octanoate is classified as a medium chain fatty acid.

It is important to recognize that the oxidation and metabolism of fatty acids is related to their carbon chain length and degree of unsaturation (Pai and Yeh, 1996). Octanoate, as a medium chain fatty acid, can enter the hepatic mitochondria relatively independently of the acyl-carnitine transport system, and then undergo β-oxidation (Miele et al., 2003). Moreover, such medium chain fatty acids are oxidized much faster than longer chain length fatty acids (Leyton et al., 1987). Other

evidence is suggestive that short and medium chain fatty acids enhance the uncoupling of oxidation in the mitochondria and subsequently provoke marked degrees of oxidative stress (Hird and Weidemann, 1966).

Fat-loading and its relationship to insulin resistance and oxidative stress is unclear. We chose to use LPON (containing octanoate as a medium chain fatty acid) and compare it to a model using longer chain fatty acids alone, namely, oleate and palmitate to fat load C3A and LX-2 cells. . As shown in this chapter, LPON increased the triglyceride content of both C3A and LX-2 cells. Palmitate was more toxic to cells compared to oleate at the same concentration.

Loading cells with oleate [0.25 mM] achieved a higher level of triglyceride accumulation as compared to cells exposed to a higher dose of octanoate [4 mM]. This would also tend to support other studies (Leyton et al., 1987) which suggest that oleate, compared to octanoate, needs regulated active transportation to allow it to enter the mitochondria (controlled metabolism). Therefore, most of the added oleate might eventually be converted to triglyceride and stored in cytoplasm as lipid droplets rather than be metabolized in the mitochondria through β-oxidation.

Selenoenzymes are known to be induced in cases where cells undergo oxidative stress; this is regarded as a protective mechanism (Moon et al., 2005). Although, LPON induced the selenoenzymes TR1 and GPX1 in both C3A and LX-2 cells, oleate or palmitate failed to show such an induction. Dissecting the LPON components established that octanoate is the main component responsible for triglyceride accumulation, as well as TR1 and GPX1 induction. Octanoate undergoes β-oxidation following its unregulated entry into mitochondria. This would markedly increase oxidative stress leading to induction of antioxidant enzymes such as TR1 and GPX1. In contrast, the mitochondrial metabolism of long chain fatty acids, such as oleate and palmitate, is controlled through regulation of entry into the organelle and, as such, these fatty acids are converted to triglyceride and deposited as lipid droplets in the cytoplasm.

Initial experiments of LPON-treated cells were done in FBS-supplemented medium but subsequently it was shown that this medium could not provide a selenium-deplete environment (chapter three). Therefore, it was important to confirm that LPON-treated cells in IT-supplemented medium (selenium-deficient medium) provide similar triglyceride, TR1 and GPX1 levels in as those cells grown in 10% FBS-supplemented medium. This confirmatory data is shown in appendix A4.1.4.

5 CHAPTER FIVE

THE EFFECT OF SELENIUM AND OXIDATIVE STRESS ON FAT-LOADED C3A AND LX-2 CELLS

5.1 Introduction

Oxidative stress is commonly defined as an imbalance in the cell in which oxidant production exceeds the cell's antioxidant capacity to neutralize these oxidants (Sies, 1997). This occurs because of either increased production rates of reactive oxygen species (ROS) or a decreased capacity of anti-oxidant systems to overcome the damaging effect of ROS (Sies, 1997). In turn, the decreased anti-oxidant capacity could be the result of either a disturbance of anti-oxidant production or of its distribution (Brenneisen et al., 2005).

An overabundance of oxidants may occur from either endogenous or external environmental sources. Commonly, ROS are by-products of cellular respiration generated by electron leakage from mitochondrial electron carriers during oxidative phosphorylation. In normal physiological processes a steady state is maintained ensuring a balance between oxidants and anti-oxidants. However, if not properly regulated, excess ROS can damage cellular lipids, proteins or DNA leading to a disruption of normal cell function and signal transduction pathways (Brenneisen et al., 2005).

Oxidative stress contributes to a range of major pathological situations and is thought to be involved in many liver disorders including alcoholic liver disease, hepatitis C and iron overload. Of particular interest with regard to liver pathology, oxidative stress has been implicated in the pathogenesis of NAFLD.

The relationship between hepatic fat accumulation and oxidative damage is poorly understood. Steatosis may be due to dysfunction in fatty acid oxidation possibly

secondary to an oxidative stress-induced dysfunction of mitochondrial β-oxidation leading to the accumulation of fat (Jaeschke et al., 2002; Koteish and Diehl, 2001). Various studies suggest that, regardless of the mechanism, the fatty liver phenotype itself is also likely to contribute to oxidative stress. The oxidative metabolism of fatty acids in steatotic liver leads to increased ROS production and thereby predisposes the hepatocytes to oxidative stress (Pessayre et al., 2002). Some reports suggest that fat can also be directly toxic to cells and induce apoptosis in hepatocytes (Schaffer, 2003). Paradoxically, other studies suggest that fat accumulation has a protective rather than a damaging effect on the hepatocyte (Listenberger et al., 2003). The type of fat which accumulates in the hepatocytes appears also to modify the hepatocyte and its susceptibility to damage by oxidative stress (Ibrahim et al., 1997; Slim et al., 1996).

In this chapter I investigated the ability of liver cells to resist oxidative damage induced by t-butyl hydroxide when the cells were pre-exposed to a range of conditions designed to influence fat-loading and selenium status. I selected t-butylhydroperoxide (t-BuOOH) as the agent to induce oxidative stress *in-vitro* because this agent that induces oxidative stress in a manner which is similar to that induced in vivo by endogenous lipid hydroperoxides (Imberti et al., 1993; Masaki et al., 1989a). t-BuOOH also damages mitochondrial and cellular membranes in cultured hepatocytes by peroxidation of membrane lipids (Masaki et al., 1989b).

Anti-oxidants neutralize the effects of oxidants and can be categorized as enzymatic and non-enzymatic agents according to their mode of action andstructure. They can also be classified as preventive, interactive or reparative anti-oxidants according to their function (Sies, 1997). Non-enzymatic compounds with direct anti-oxidant actions include ascorbate, carotenes, flavonoids, glutathione, and tocopherols. The most common anti-oxidant enzymatic defense systems are superoxide dismutases, catalase, thioredoxin, thioredoxin reductases, and glutathione peroxidases (Sies, 1997). Because micronutrient elements such as selenium, zinc, manganese and copper are essential integral elements of many of the enzymes that have anti-oxidants action, there is much interest in the supply of these micronutrients in ant-oxidant

defense. (Brenneisen et al., 2005). With specific reference to selenium, TR1, TR2 and GPX1 are regarded as the most the important anti-oxidant selenoenzymes.

Whether enzymatic or non-enzymatic, an anti-oxidant may have a protective, interventional and/or repair role (Sies, 1997). Examples of protective anti-oxidants are melanin which protects the skin from ultra-violet rays and cytochrome oxidase, which carries out cellular oxygen reduction with minimal release of superoxide or other radicals. Metal chelation is an additional major mechanism which limits lipid peroxidation and DNA damage (Sies, 1997).

Humans acquire selenium mainly from grains, cereals and meat and an adequate supply of the trace element is required to ensure maximal synthesis of selenoenzymes (Prabhu et al., 2002).

As I have observed in the previous chapter, selenium supplementation allows TR1 and GPX1 to be expressed optimally in cultured C3A and LX2 cells. In this chapter I investigate whether selenium supplementation could protect cultured hepatocytes and stellate cells from an oxidative stress induced by t-BuOOH. In addition, the effect of fat-loading cells on their susceptibility to oxidative damage induced by t-BuOOH was also investigated.

5.2 Methods

General protocols:

For the experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in sections 2.2.1. In order to modify the selenium status of the cells they were passaged and re-plated into 35 mm 6-well plates at a density of 100,000 cells per well and further culture was in IT medium, unless otherwise stated. After 24 hr incubation, treatments were started as described below. The LDH contents of cells and collected media were measured as described in section 2.2.4 to determine percentage LDH retention as a measure of cell damage/viability.

Protocol 5.2.1: One group of cells was pre-treated with selenium [100 nM] for 48 hours. C3A and LX-2 cells were then exposed to various doses of t-BuOOH (0-1000 µM) for a further 24 hours. The LDH retention of cells were measured as described in section 2.2.4

Protocol 5.2.2: C3A cells were pre-treated with selenium [100 nM], LPON (Lactate [20 mM], Pyruvate [1 mM], Octanoate [4 mM], NH_4Cl [4 mM]) or selenium plus LPON for 48 hours. C3A cells were then exposed to various doses of t-BuOOH ranging from a sub-lethal dose of 200 µM rising to a toxic concentration of 1000 µM for a further 24 hours. In further experiments, oleate [0.25 mM], either with or without selenium [100 nM], was also added for 24 hours as a pre-treatment prior to addition of t-BuOOH. (These conditions are based upon results from appendix A4.1.2 which showed that an incubation time of 24 hours with oleate treatment was sufficient for triglyceride accumulation).

Protocol 5.2.3: C3A cells were pre-treated with either lactate/pyruvate [20 mM] / [1 mM], octanoate [4 mM], NH_4Cl [4 mM], oleate [0.25 mM] plus or minus selenium [100 nM] for 48 hours. Various doses of t-BuOOH were added (200µM-3200µM) for a further 24 hours prior to assessment of LDH retention.

Protocol 5.2.4: C3A cells were pre-treated with either oleate [0.25 mM], octanoate [4 mM], LPOleateN (lactate/pyruvate [20 mM] /[1 mM] oleate [0.25 mM], NH_4Cl [4 mM]), or LPON. Experiments were carried out with or without selenium [100 nM] for 48 hours. T-BuOOH in various doses was added (200µM-3200µM) for a further 24 hours prior to assessment of LDH retention.

5.3 Results

5.3.1 The protective effect of selenium in C3A and LX-2 cells during t-BuOOH-induced oxidative stress

A preliminary experiment was conducted to determine the concentration of t-BuOOH required to induce damage of C3A cells as assessed by LDH release from cells. The viability of cells was assessed by monitoring percentage LDH retention in these cells. It was concluded that concentrations of t-BuOOH between 0-3200 [µM] were able to induce various degrees of damage to C3A cells depending on their initial selenium status (data not shown).

To investigate any protective effect of selenium on C3A and LX-2 cells undergoing t-BuOOH-induced oxidative stress, protocol 5.2.1 was followed.

The susceptibility of both C3A and LX2 cells to damage by t-BuOOH was clearly dependent on their selenium status (figure 5.1). Selenium-supplemented LX2 and C3A cells showing marked protection from damage induced by t-BuOOH.

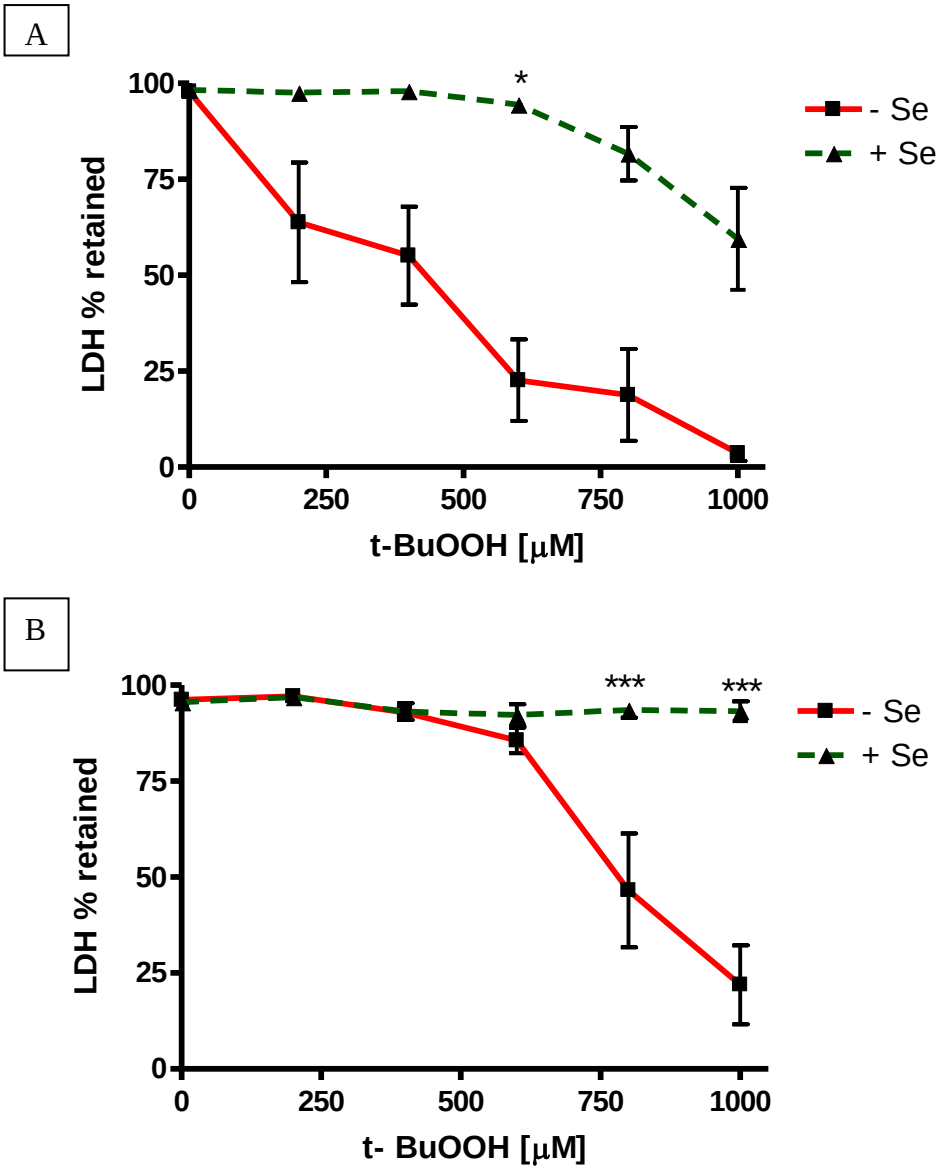


Figure 5.1: The effect of selenium on the viability of oxidatively stressed (A) C3A and (B) LX-2 cells: Both cell types were pre-treated with or without selenium for 48 hours. Cells were then exposed to increasing concentrations of t-BuOOH for a further 24 hours. Data represent the mean ± standard error (± SEM), n=3 experiments for both C3A (A) and LX2 (B) cells. Each experiment was done using triplicate wells. * $p<0.05$, *** $p<0.001$ significant difference between Se- and Se+ cells. Two-way ANOVA; Bonferroni post-test.

5.3.2 The effects of selenium status and fat-loading on C3A cells viability during t-BuOOH-induced oxidative stress

To investigate both the effects of selenium status and fat-loading on the ability of C3A cells to resist oxidative damage induced by t-BuOOH Protocol 5.2.2 was followed.

Oleate was chosen to be part of the pre-treatment because LPON had been shown to increase the level of TR1 (see section 4.3.1.2) while oleate-treatment of cells failed to affect the level of TR1 suggesting oleate treated cells were not undergoing a marked oxidative stress.. It was hypothesized that different fatty acids might have different effect on hepatocyte function and their susceptibility to oxidant damage.

As shown in figures 5.2A and figure 5.2B selenium pre-treated cells were highly resistant to damage induced by t-BuOOH when compared with selenium deficient cells. This protective effect was apparent irrespective of their fat loaded state using LPON (figure 5.1A) or oleate (figure 5.1B).

Panel 5.2C represent a combination of the data given in panel A and B together with data from selenium deficient or selenium supplemented cells that had not been treated with fatty acids. This allows a comparison of LPON and oleate treatments in selenium replete and selenium deficient states. There was no significant ($p>0.05$) effect of either oleate or LPON treatment on the susceptibility of the cells to t-BuOOH-induced damage; this was true irrespective of the selenium status of the cell. However, it should be noted that there was a general trend for LPON and oleate-treated cells to show more susceptibility to damage by t-BuOOH than cells that were not fat loaded.

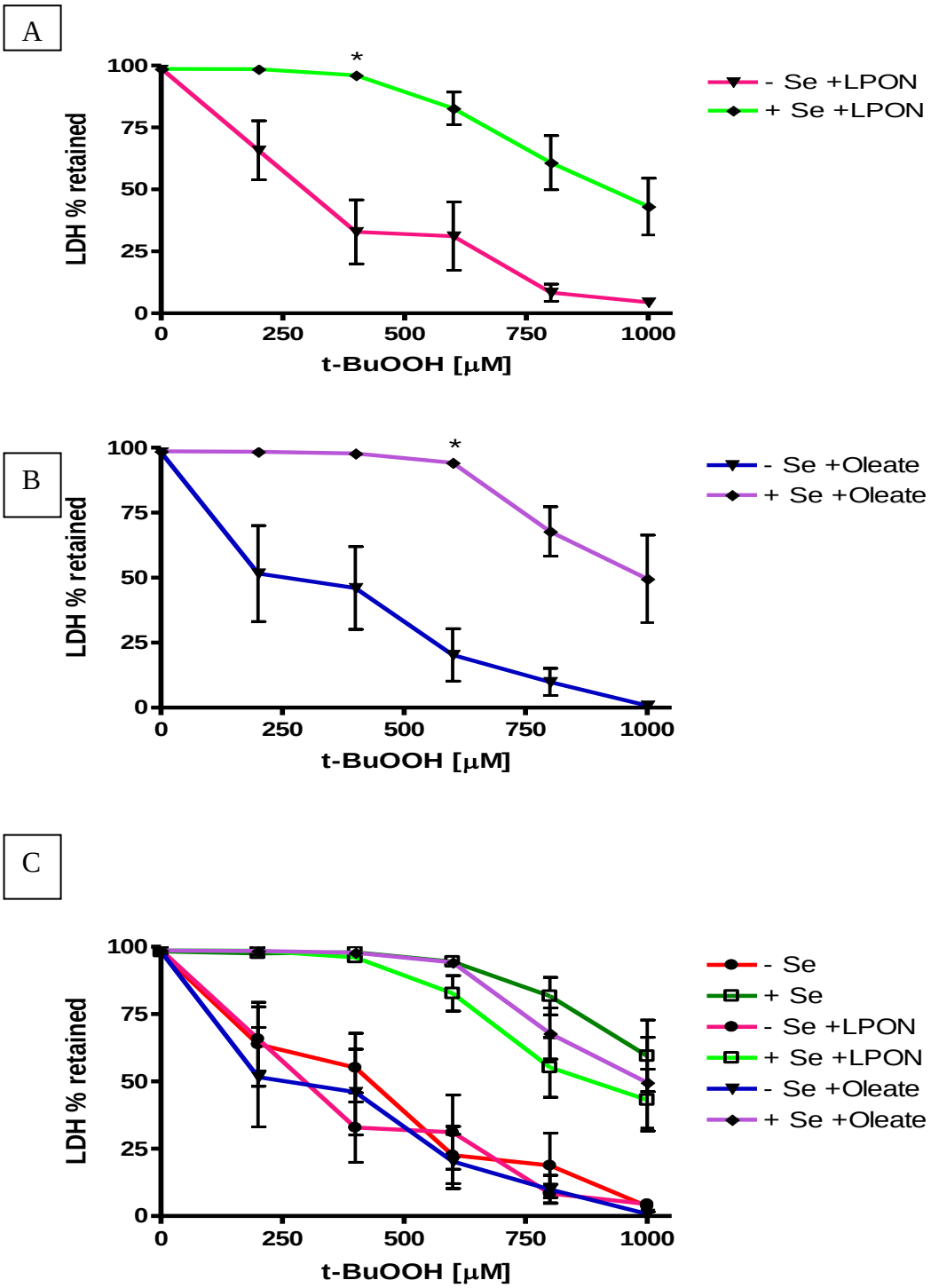


Figure5.2: The effect of selenium and fat loading on the viability of C3A cells exposed to t-BuOOH

C3A cells were pre-treated with either (A) LPON for 48 hours (plus or minus selenium) (B) oleate for 24 hours (plus or minus selenium) (C) comparison of both pre-treatments to control (un-treated) cells. Cells were then exposed to different concentrations of t-BuOOH for further 24 hours and LDH retention assessed. Data represent the mean ± standard error (± SEM), n=3 experiments each experiment being done in triplicate. * $p<0.05$ compared to selenium deficient cells. Two-way ANOVA with Bonferroni post-tests was used for statistical analysis.

5.3.3 The effect of individual LPON components on C3A cell viability undergoing oxidative stress

To determine if any of the LPON components may be harmful to C3A cells, protocol 5.2.3 was followed.

As shown in figure 5.3A and figure 5.3B there was no significant difference between various pre-treatments used irrespective of the selenium status. However, as in figure 5.2C, there was a general trend for LPON treated cells to be more susceptible to damage induced by t-BuOOH.

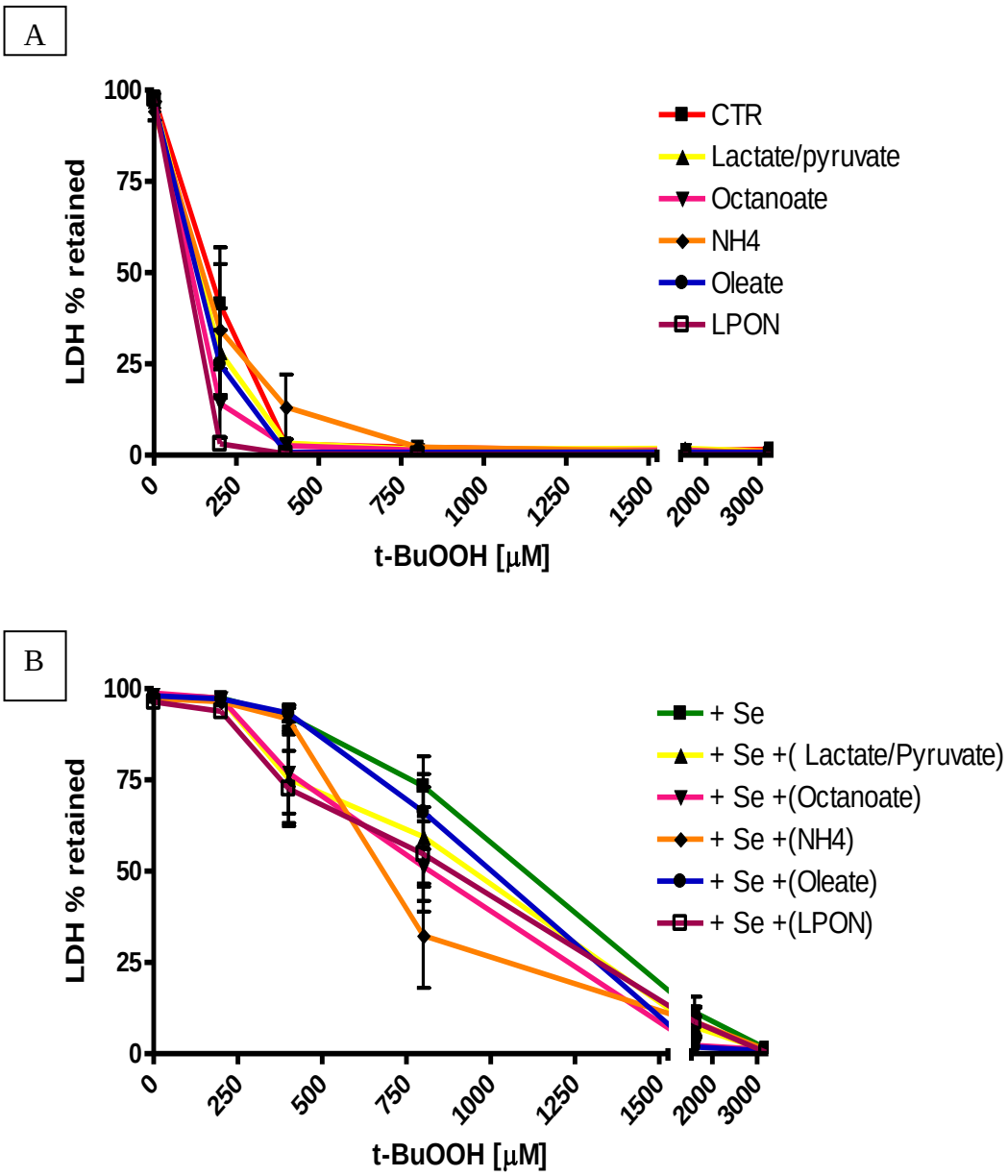


Figure 5.3: The effect of individual components of LPON on the viability of oxidatively stressed C3A cells in the (A) absence or (B) presence of selenium

C3A cells were pre-treated with individual components of LPON and oleate in the (A) absence or (B) presence of selenium [100 nM] for 48 hours. The cells were then exposed to different doses of t-BuOOH for a further 24 hours. The LDH retention of cells was then assessed as described in section 2.2.4. Data represent the mean ± standard error (± SEM), n=3 experiments for both A and B, each experiment done using triplicate incubations.

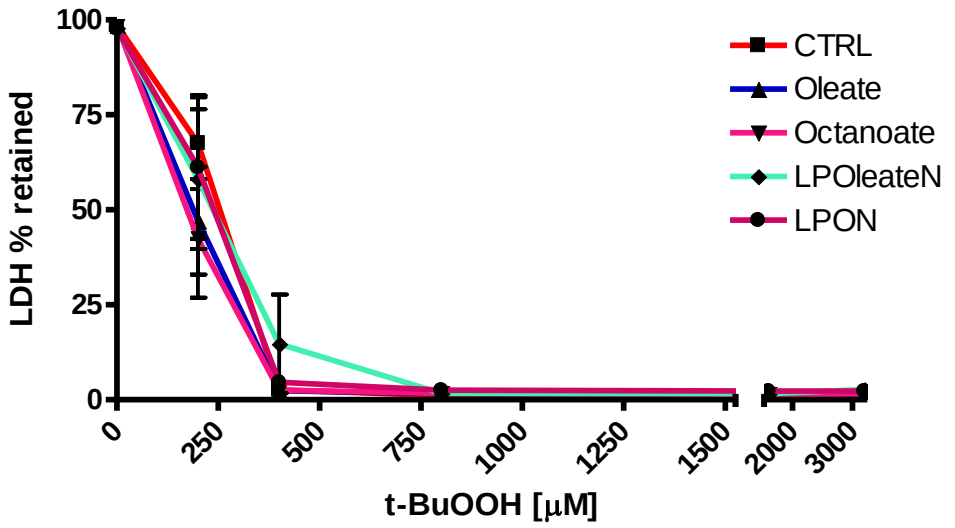
5.3.4 The effect of a medium combining lactate, pyruvate and ammonia with oleate (LPOleateN) on C3A cell viability undergoing oxidative stress induced by t-BuOOH.

It was decided to investigate the effect of replacing octanoate with oleate as the fatty acid component of LPON. For this purpose, protocol 5.2.4 was followed.

As shown in figure 5.4A, in the absence of selenium, all pre-treatment regimens appeared to have no effect on the susceptibility of cells to t-BuOOH.

When selenium-supplemented cells were used again no significant ($p>.0.05$) effect of any pre-treatment was apparent on cell viability following exposure to t-BuOOH. However it seems clear that again a general trend was apparent for each of the components of LPON and oleate to increase the susceptibility of these cells to damage by t-BuOOH (figure 5.4B).

A



B

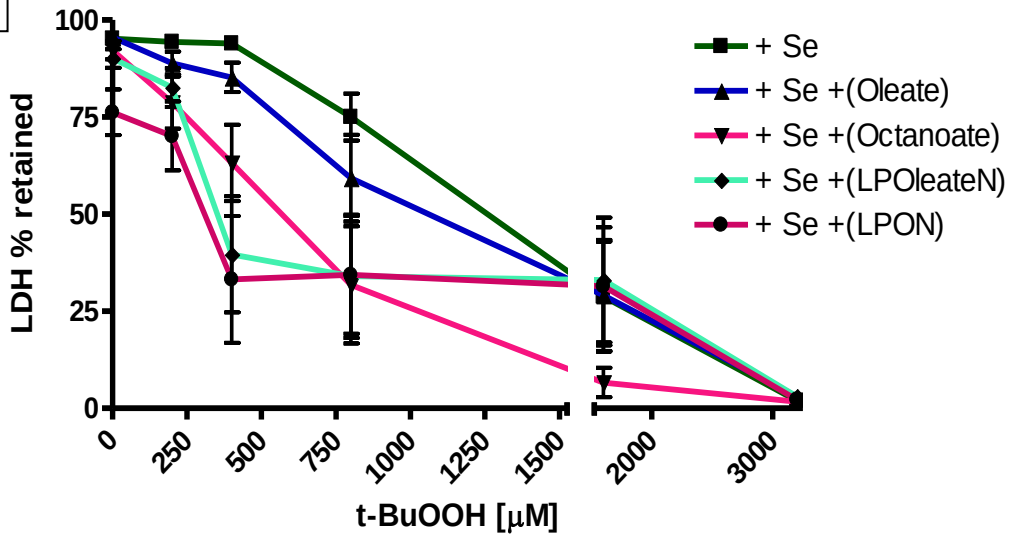


Figure 5.4: The effect of fatty acids and LPON on the susceptibility of C3A cells to damage from t-BuOOH in (A) absence or (B) presence of selenium. C3A cells were pre-treated with either oleate, octanoate, LPON or LPOleateN in the presence or absence of selenium [100 nM] for 48 hours. The cells were then exposed to different doses of t-BuOOH for a further 24 hours. The percentage LDH retention was then assessed as described in section 2.2.4. Data represent the mean ± standard error (± SEM), n=3 each experiment done using triplicate incubations.

5.4 Conclusion and Discussion

Conclusion:

Our findings provide support the following conclusions:-

1. In culture, selenium supplementation can provide hepatocytes with marked protection from damage caused by oxidative stress induced by lipid hydroperoxides. From the work presented in chapter three, such protection is likely to be due to the induction of a range of selenoenzymes with antioxidant action including the families of GPX and TR. If this reflects the situation *in vivo* it suggests that low selenium status, possibly arising through relative lack of selenium in the diet, would predispose hepatic tissue to oxidative injury in humans.

2. The data presented show that fat loading cells with either oleate or LPON provides no protection from oxidative stress induced by t-BuOOH.

3. Whilst there was also no conclusive data to suggest that LPON predisposes cells to injury from t-BuOOH, the general trend in all experiments indicated that LPON could indeed compromise the ability of cells to resist oxidative stress.

Discussion

Oxidative stress is a major destructive process involved in the pathogenesis of many disorders. The destructive process derives from an array of molecules produced during oxidative stress. Different molecules have different potential to damage other cellular components. The shorter the half life of a molecule, the more haphazard its action and the greater its destructive potential will be. For example, hydroxyl radicals (OH•) have a very short half life of 10^{-9} seconds and therefore a major destructive ability. On the other hand, lipid peroxidation products, such as 4-hydroxy-2,3-nonenal (HNE) and 4-hydroxy-2,3-alkenals (HAK), have a longer half life and have less damaging potential (Parola and Robino, 2001).

The consequences of oxidative stress and production of ROS modify the binding of ligands to a range of membrane receptors and cause peroxidation of membrane lipids and DNA damage (Parola and Robino, 2001). Reports on the effect of mild oxidative stress include proteolysis of many proteins and enzymes involved in cell function, especially the activities of anti-oxidant enzymes including superoxide dismutase and glutathione peroxidases (Grune et al., 1995). Other studies suggest that cells undergo apoptosis at intermediate levels of oxidative stress whilst massive oxidative stress induces necrosis of the cells (Parola and Robino, 2001). Morphologically, the hepatocyte becomes swollen and the mitochondria suffer damage when subjected to an oxidative stress(Gonzalez-Flecha et al., 1993) and this is thought to be at least in part due to the production of lipid hydroperoxides. On this basis, t-butyl hydroperoxide was used in our experiments because it is a lipid hydroperoxide that is widely used as an agent to induce a controlled oxidative stress in cultured cells (Imberti et al., 1993).

Oxidative stress is a major pathogenic factor pathway in liver diseases including hepatitis C (Choi and James Ou, 2006). In fatty liver, oxidative stress is considered to promote the inflammatory process by enhancing the production and activities of chemokines and inflammatory cytokines to recruit infiltrating inflammatory cells (Dong et al., 1998).

The findings of the experiments presented in this chapter demonstrate that in cultured hepatocytes (C3A cells) adequate selenium status provides a major protective element against oxidative stress induced by lipid hydroperoxides. Moreover, selenium can provide significant protection from oxidative stress to both normal and fat loaded C3A cells. These beneficial effects of selenium pre-treatment do not become apparent unless a prolonged (>24h) pre-treatment period with selenium is performed (Rafferty et al., 1998). This is because the ability of selenium to prevent oxidative damage requires the synthesis of selenoproteins. For this reason, in our experiments, cells were pre-treated with selenium for 48 hours to ensure selenoenzyme expression was maximal.

Similar experiments were also conducted on LX-2 cells with findings similar to those found in C3A cells. This suggests that selenium-repletion might alleviate the effect of oxidative stress in liver tissue regardless of the cell type. This observation is important since antagonising the oxidative stress in multiple cells types is one of the key issues in protecting hepatic tissues from damage. The relationship between oxidative stress and fibrogenesis is complex and not fully understood. Several studies suggest that an indirect effect of oxidative stress on hepatocytes and other inflammatory cells, such as Kupffer cells and neutrophils, is the main regulator of activation of hepatic stellate cells (Giuliana et al., 1998). Other studies claim that direct effects of oxidative stress on stellate cells leads to their activation with release of collagen and progress to liver fibrosis (Houglum et al., 1997).

It is clear that protecting the hepatocytes from the release of different inflammatory cytokines or protecting the stellate cells themselves are both potential mechanisms to moderate the fibrotic process. The key issue is the limitation of the fibrotic process in both hepatocytes and stellate cells and this is investigated in chapter six.

We were unable to demonstrate any significant effect of fat loading on the ability of cells to resist damage by t-BuOOH. However it is worth noting that there was a clear trend for LPON-treated cells to be more susceptible to oxidative damage than untreated cells irrespective of the cell's selenium status. Further dissection of the individual components of LPON revealed that each of the components of LPON may predispose cells to damage by t-BuOOH.

A similar but less marked trend was also observed in oleate-treated cells. The failure to produce a significant effect of LPON or oleate pre-treatments in our experiments was in part due to the poor reproducibility of data between experiments (as illustrated by the large SEM of the data points in figures 5.3 and 5.4). Further experiments are required to investigate the effects of LPON and oleate on C3A cells, including direct measurement of oxidative stress in these systems. These trends might be explained by the fact that short chain fatty acids like octanoate can enter the hepatic mitochondria relatively independently of the carnitine transport system, and directly

undergo β-oxidation (Miele et al., 2003). Moreover, such medium chain fatty acids are oxidized much faster than other longer chain length fatty acids (Greenberger et al., 1966; Leyton et al., 1987) leading to a greater oxidative stress. Other evidence is suggestive that short and medium chain fatty acids enhance the uncoupling of oxidation in the mitochondria and subsequently provoke oxidative stress (Hird and Weidemann, 1966).

It should be noted however that one study using HepG2 cells loaded with oleate and/or palmitate as a model for NAFLD, was unable to demonstrate increased oxidative stress following fat-loading (Gomez-Lechon et al., 2007).

Finally the data presented here provide strong evidence for an important antioxidant role for selenium in hepatocytes and hepatic stellate cells. If this occurs in vivo it suggests that low selenium status, possibly arising through relative lack of selenium in the diet, would predispose hepatic tissue to oxidative injury in humans. As such, a low selenium status may be a contributory factor to the pathogenesis of both alcoholic and no-alcoholic liver disease.

6 CHAPTER SIX

THE EFFECT OF SELENIUM ON α-1 PRO-COLLAGEN EXPRESSION OF C3A AND LX-2 CELLS

6.1 Introduction

The hepatic stellate cell (HSC) is a major fibrogenic cell type that contributes to collagen accumulation during chronic liver disease. In healthy liver, hepatic stellate cells act as a reservoir of vitamin A and are believed to be responsible for the maintenance of a steady hepatic blood flow (Kershenobich Stal and Weissbrod, 2003). The immortalized hepatic stellate cell line, LX-2, was chosen for this study because it has retained features of cytokine- and fibrogenesis-signalling which are similar to those seen *in-vivo*. LX-2 cells are thus widely regarded as a suitable model for cell culture-based studies that are designed to investigate human hepatic fibrosis (Xu et al., 2005).

Various trials have been undertaken to try to inactivate hepatic stellate cells through various mechanisms, including apoptosis and causing reversion to an inactive phenotype. It is believed that such mechanisms are important in liver regeneration and resolution (Issa et al., 2001; Wright et al., 2001). One strategy focused on the direct antagonism of fibrosis gene expression by the use of agents such as halofuginone which inhibit collagen synthesis (Gnainsky et al., 2004). Other strategies have focused on moderating the oxidative stress associated with the activation process or relieving the effect of activating cytokines and/or associated signaling molecules. One important class of anti-oxidants, namely the selenoenzymes, have been largely ignored in relation to the pathogenesis of NAFLD and the associated fibrosis. .Selenium is very effective in preventing oxidative stress in several tissues (Kim et al., 2004; Santosh et al., 1999) through promoting the synthesis of selenoenzymes (glutathione peroxidases and thioredoxin reductases)

that have powerful anti-oxidant functions. In this chapter I have examined the possibility that selenium may limit the fibrogenesis in NAFLD.

The mechanism by which α-1 pro-collagen is released by hepatic stellate cells is not fully understood. Suggested factors affecting the production of α-1 pro-collagen are either a direct action of oxidative stress or stimulation of stellate cells by cytokines (released from nearby injured cells) which then can act in a paracrine fashion (Svegliati Baroni et al., 1998). In Chapter five it was found that selenium could prevent damage caused by oxidative stress both in the hepatocyte C3A cell line and the hepatic stellate LX-2 cell line. It was thus of interest to investigate if selenium status could modify, fibrogenesis and more specifically the expression of α-1 pro-collagen in C3A and LX2 cells. To the best of our knowledge, no study has been reported on the effect of selenium status on the expression of α-1 pro-collagen.

Although the literature stresses the importance of the hepatic stellate cells in liver fibrosis, the cell type(s) responsible for the fibrotic process remains controversial. For example, it is possible that the hepatic stellate cells might have a hepatocyte origin (epithelial phenotype). Moreover, the transition of epithelial cells to a myofibroblasts cell type is a well-described phenomenon in various fibrotic disorders in other tissues such as lung (Willis et al., 2006) and kidney (Vongwiwatana et al., 2005; Zeisberg and Kalluri, 2004).

The aims of the experiments reported in this chapter were to study the expression of α-1 pro-collagen mRNA in both C3A and LX-2 cells and to investigate the effect of selenium status, conditioned medium or co-culture with C3A cells on the expression of α-1 pro-collagen in LX-2 cells.

6.2 Methods

General Protocol: For the purpose of experiments reported in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in section 2.2.1. The cells were passaged and re-plated into 6-well plates at a density of 100,000 cells per well. After a 24 hr incubation treatments were started as described below. α-1 Pro-collagen mRNA was measured as described in section 2.2.10.

Optimization of the α-1 pro-collagen probe and primer concentrations for analysis on Taqman Real-Time PCR was dependent on two principal factors:

1. The concentrations of primers and probe that would give the lowest C_T value (most sensitive to detect low conc. of mRNA)
2. The concentrations of primers and probe to give the least variance in triplicate results (i.e. best precision)

I concluded from preliminary experiments that the best forward primer /reverse primer/ probe concentrations were 250/250/100 nM (final concentrations), respectively.

Protocol 6.2.1: LX-2 cells were therefore incubated for 24 hours in either 0.2 %BSA-supplemented medium or IT-supplemented medium. The two sets of cells were then treated with TGF-β1 [2.5 ng/ml] for 24 hours and the cellular mRNA collected from these cells was quantified as described in section 2.2.10.

Protocol 6.2.2: LX-2 cells were incubated in IT-supplemented medium for 24 hours, and then pre-treated with selenium [100nM] or LPON for 48 hours. Tertiary-BuOOH [200 µM], TGF-β1 [2.5 ng/ml] and fresh treatments of selenium and LPON were then added to the cells for a further 24 hours. The cells were then collected and mRNA extracted and assessed as described in section 2.2.10.

Protocol 6.2.3: C3A cells were incubated in IT medium for a preliminary period of 24 hours. The cells were then treated with LPON and selenium [100 nM] for 48 hours. C3A cells were subsequently recharged with fresh treatments of LPON, selenium, t-BuOOH [200 µM] and/or oleate [0.25 mM] for a further 24 hours. The cells were then washed twice and treated with IT medium for a further 24 hours. These various conditioned media from C3A cells were then collected for addition to LX-2 cells. TGF-β1 [2.5 ng/ml] and the various conditioned media were added to LX-2 cells for a final 24 hours. After this 24 hour period, LX-2 cells were then harvested and pro-collagen mRNA extracted and assessed as described in section 2.2.10.

Protocol 6.2.4: C3A cells were cultured in cell culture inserts and incubated in IT medium for a preliminary 24 hour period. The cells were then treated with LPON +/- selenium [100 nM] for 48 hours before further treatment with IL-8 [500pg/ml], fresh amounts of LPON, oleate [0.25 mM] or t-BuOOH [200 µM] +/- selenium [100 nM] for a further 24 hours. The cells were then washed twice and treated with IT medium. Corresponding wells of LX-2 cells were ready to receive the cell culture inserts from the various C3A cell treatments. TGF-β1 [2.5 ng/ml], IL-8 [500pg/ml] and C3A cell culture inserts were then added to LX-2 cells for a final 24 hour incubation. LX-2 cells were then harvested and α-1 pro-collagen mRNA extracted and measured as described in section 2.2.10.

Protocol 6.2.5: C3A cells were divided into two groups, one group were cultured in IT medium and the second was cultured in ITS for 72 hours. Both groups were further divided into subgroups in order to allow treatment with LPON or oleate [0.25 mM]. After 48 hours treatment, the respective media were replaced to allow treatment with TGF- β1 [2.5 ng/ml] for a further 24 hours. The cells were then collected and α-1 pro-collagen mRNA measured as described in section 2.2.10.

6.3 Results

6.3.1 The expression of α-1 pro-collagen mRNA in LX-2 cells:

6.3.1.1 Optimization experiment

6.3.1.1.1 Comparison of the expression of α-1 pro-collagen mRNA in LX-2 cells in IT-medium versus 0.2% BSA-supplemented medium

The majority of experiments conducted in this thesis were designed to investigate the effects of selenium status. To achieve a selenium-deficient state in cells that remained viable it was necessary to use IT medium as described in Chapter 3. Since many published studies have used cells cultured with 0.2% BSA rather than IT, a first step was to determine if cells grown in IT medium produced measurable amounts of α-1 pro-collagen mRNA and also determine if the levels were similar in IT versus BSA-treated cells. Also the effects of LPON and selenium addition on α-1 pro-collagen mRNA were investigated according to protocol 6.2.1.

As shown in figure 6.1, the expression of α-1 pro-collagen mRNA in LX-2 cells was similar in both settings (0.2%BSA versus IT-medium). Neither LPON nor selenium treatments had any effect on the α1- pro-collagen mRNA levels whether the cells were grown in 0.2%BSA-supplemented or IT-supplemented medium.

The treatment of LX-2 cells with TGF-β1 for 24 hours gave a significant two-fold increase in expression of α 1-pro-collagen mRNA in both settings.

Further experiments were done comparing shorter incubation period of TGF- β1 but it was only when incubated for 24 hours where a significant two-fold increase in expression of α 1-pro-collagen mRNA was seen as described in appendix A6.2.1.

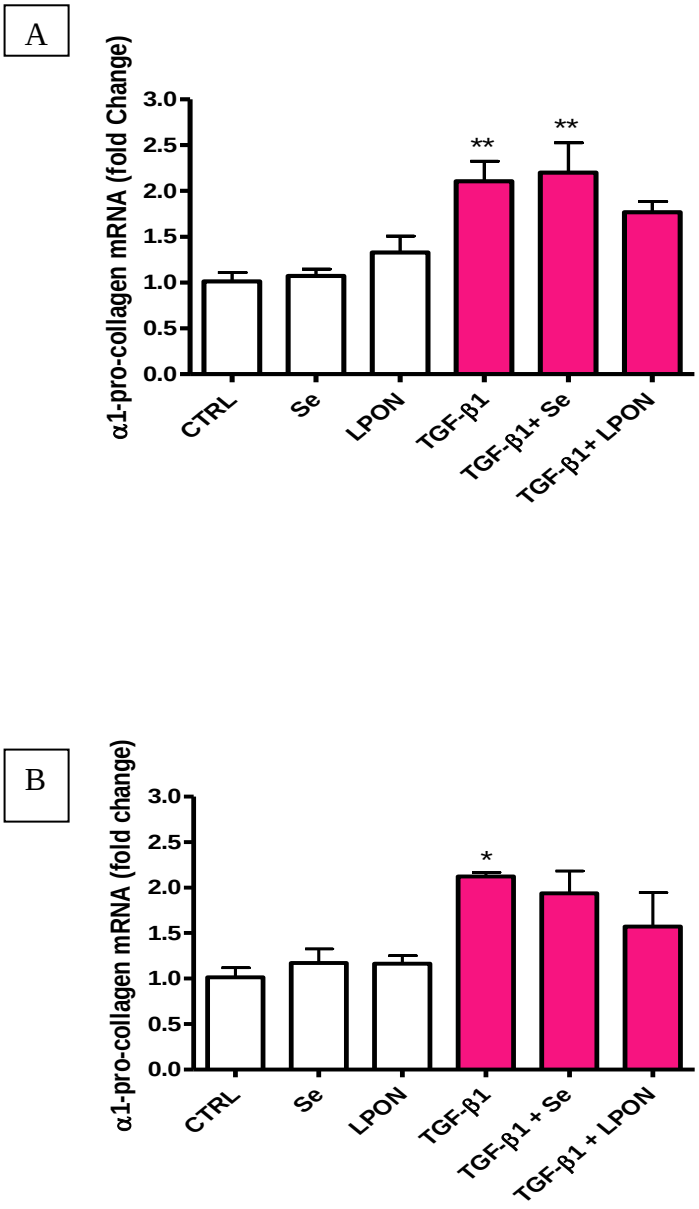


Figure 6.1: The effect of various treatments on the expression of α1-pro-collagen mRNA in (A) cells grown in 0.2% BSA or (B) cells grown in IT medium

LX-2 cells were pre-treated where indicated with selenium [100 nM] or LPON for 48 hours. TGF-β1 [2.5 ng/ml] was then added for further 24 hours. Panel A represents cells incubated in 0.2%BSA supplemented medium. Panel B represents cells incubated in IT supplemented medium. Data are the mean ± standard error (± SEM) for three experiments (n=3). Triplicate wells were used for each experiment. * $p<0.05$, ** $p<0.01$ compared to corresponding controls (CTRL) grown in either 0.2%BSA-supplemented medium (panel A), or grown in IT-supplemented medium (panel B).

6.3.1.2 Hypothesis-driven experiments

6.3.1.2.1 The effect of selenium and TGF-β1 on the expression of α-1 pro-collagen mRNA in LX-2 cells pre-treated with LPON or t-BuOOH

Because the role of oxidative stress in regulating the expression of α-1 pro-collagen is somewhat controversial, we investigated the effect of t-BuOOH, an agent known to induce oxidative stress, on α-1 pro-collagen mRNA production in LX-2 cells. The effect of t-BuOOH was compared with that of selenium and LPON addition according to protocol 6.2.2.

As shown in figure 6.2A, neither selenium nor LPON had any effect on the basal expression of α-1 pro-collagen mRNA in LX-2 cells. This confirmed what was reported earlier (see figure 6.1). Surprisingly, t-BuOOH-treated cells showed a significant decrease in their α-1 pro-collagen mRNA levels, to about 10 % of that seen in control cells. Further experiments were done to dissect that effect and are shown in the appendix.. It was revealed that t-BuOOH showed a dose-dependent inhibition of α-1 pro-collagen mRNA (appendix A6.2.2). Furthermore, this effect of t-BuOOH was also observed on gene products that were both related (*lysyl oxidase)* and unrelated *(steroid suphatase)* to α-1 pro-collagen mRNA expression (appendix A6.2.2).

TGF-β1-treated cells produced a two-fold increase of α-1 pro-collagen mRNA levels (figure 6.2B) in control cells but TGF- β1 was unable to initiate a stimulatory response on α-1 pro-collagen mRNA in the presence of t-BuOOH. Selenium appeared to ameliorate the effect of t-BuOOH, both in the absence (6.2A) or presence (6.2B) of TGF- β1.

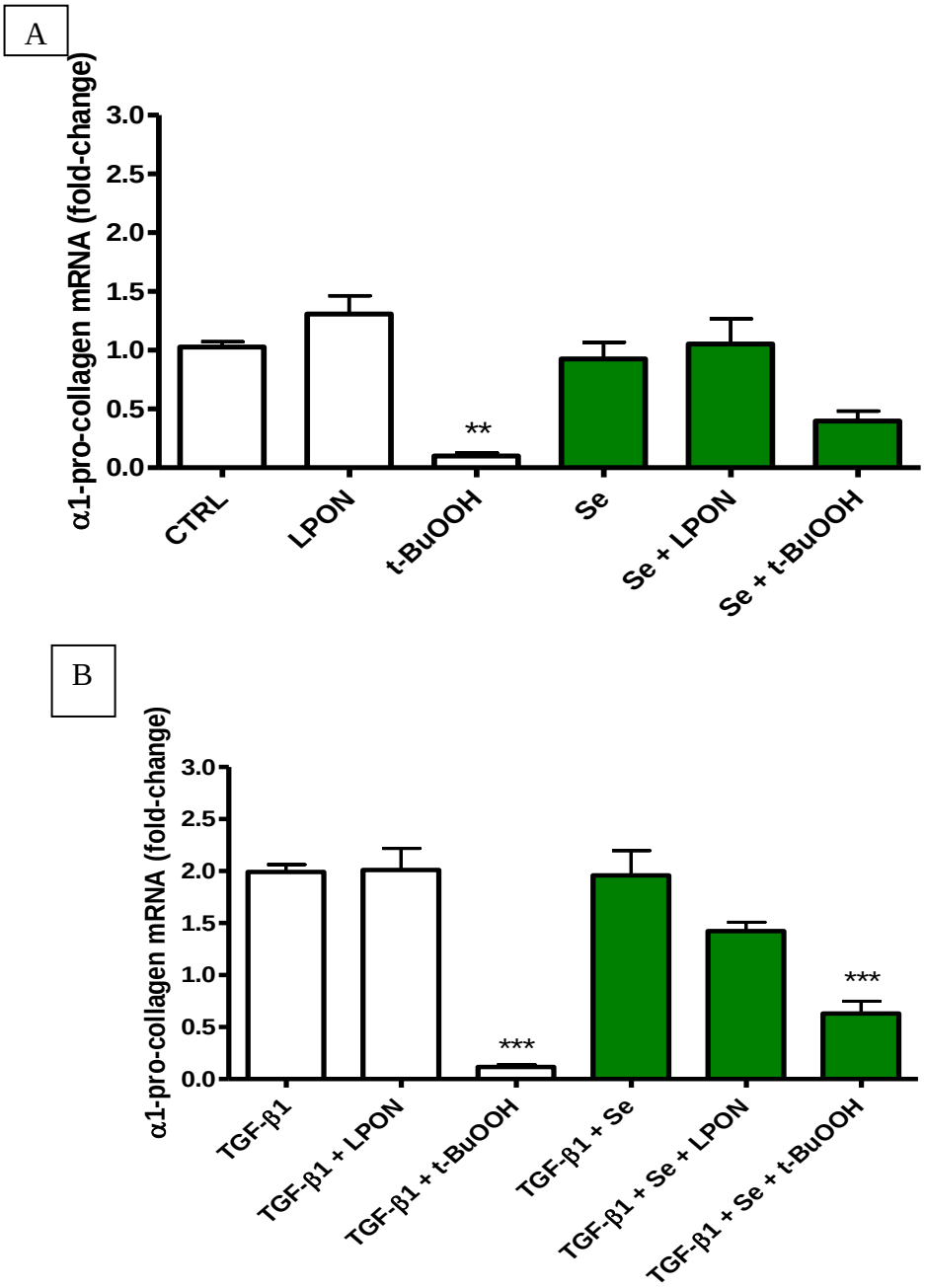


Figure 6.2: The effect of LPON and t-BuOOH pre-treated LX-2 cells on the expression of α-1 pro-collagen in the (A) absence or the (B) presence of TGF- β1 LX-2 cells were pre-treated with selenium [100 nM], LPON for 48 hours. The cells were then treated with a sub-lethal dose of t-BuOOH [200 µM], together withTGF-β1 [2.5 ng/ml] for a further 24 hours. The cells were then collected and assessed as described in section 2.2.10. Data are the mean ± standard error (± SEM) of three experiments (n=3), each experiment done in triplicate wells. ** $p<0.01$, *** $p<0.001$ compared to corresponding controls, CTRL in panel A and TGF-β1-treated cells in panel B.

6.3.1.2.2 The effect of conditioned media from C3A cells on the expression of α-1 pro-collagen mRNA in LX-2 cells

The pathogenesis of NAFLD most likely involves the interaction of different cell types. Thus an important mechanistic pathway underlying its pathophysiology may involve hepatocyte fat-loading, inflammation and/or injury affecting the activation of the hepatic stellate cells by a paracrine action.

Increased expression of α-1 pro-collagen may be dependent on either a direct oxidative stress effect on hepatic stellate cells or an indirect one caused by cytokine release from adjacent stressed hepatocytes. We thus investigated whether conditioned media from C3A cells obtained after various states of fat-loading, oxidative stress or selenium treatment had an effect on α-1 pro-collagen mRNA levels in LX2 cells according to protocol 6.2.3.

Conditioned medium from C3A cells treated with LPON and oleate had no significant effect on the α-1 pro-collagen expression in LX-2 cells (Fig 6.3A). Conditioned medium from selenium-supplemented C3A cells produced a small (about 25%) but significant ($P<0.05$) decrease in LX-2 α-1 pro-collagen mRNA levels when compared to control (conditioned LX-2 cells). This was true also of selenium replete C3A cells treated with oleate. Conditioned medium of selenium-replete C3A cells treated with LPON or t-BuOOH had no effect on the expression of LX-2 α-1 pro-collagen mRNA.

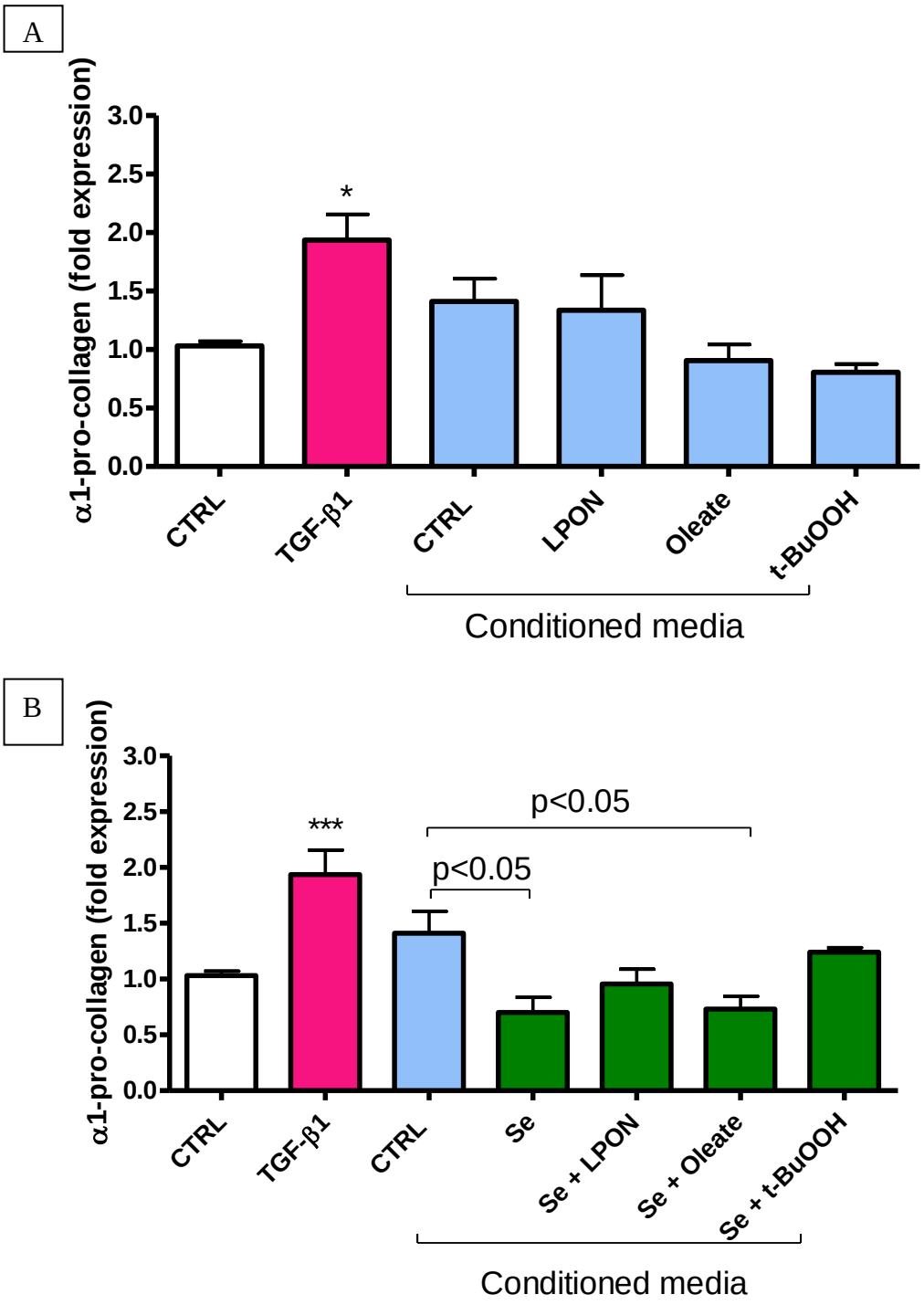


Figure 6.3: The effect of conditioned media from C3A cells on LX-2 cell expression of α-1 pro-collagen in the absence (A) or presence (B) of selenium. C3A cells were treated with LPON, oleate [0.25 mM], t-BuOOH [200 µM] +/- selenium [100 nM] for 72 hours. The cells were then treated with fresh IT medium for a final 24 hours. The conditioned media were then collected and applied to LX-2 cells. TGF-β1 [2.5 ng/ml] 24 hour-treated LX-2 cells were used as a positive control. Data represent the mean ± standard error (± SEM) of three experiments performed with triplicate incubations (n=3). * $p<0.05$, *** $p<0.001$ compared to untreated LX-2 cells (CTRL).

The cytokine contents of the conditioned media collected from the C3A cells in above experiment was also assessed (see section 6.3.1.2.2). These cytokines included TNF-α, IL-12, IL-6, IL-10, IL-1β and IL-8 and were kindly measured by Dr Suzanne Mackenzie as described in section 2.2.13 using a commercial cytometric bead assay. However, only IL-8 levels were found to be detectable in the culture medium using this assay.

As shown in figure 6.4, selenium-treatment of C3A cells decreased the level of IL-8 by about 50 %. Oleate-treated C3A cells showed an increased IL-8 level (~ 50 %) and selenium supplementation was able to reduce the IL-8 concentration in oleate treated cells also (~ 10 %).

The effect of further treatments on IL-8 release was tested using t-BuOOH and LPON (data not shown). LPON treatment was of no effect on the IL-8 release regardless of the selenium status. Intriguingly, in selenium-deficient cells, t-BuOOH treatment reduced the IL-8 level by 50 %. Furthermore, t-BuOOH plus selenium treatment was not significantly different from untreated cells.

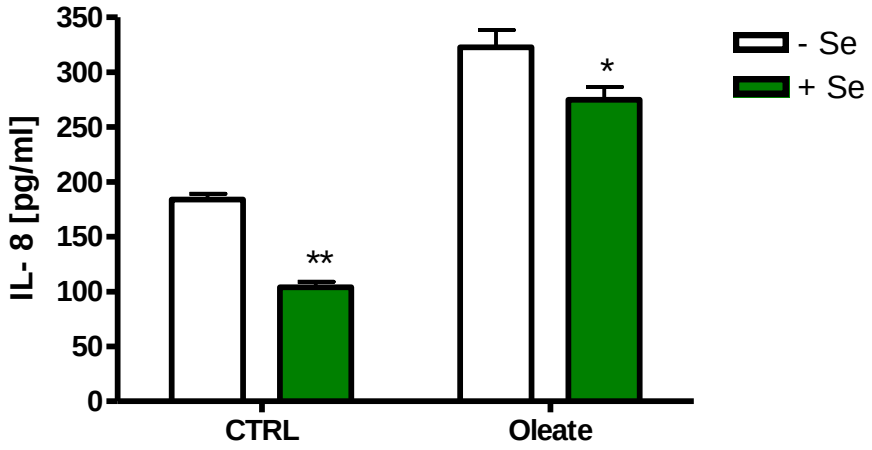


Figure 6.4: The effect of selenium status and oleate loading on the (IL-8) secretion by C3A cells. IL-8 levels of conditioned media from C3A cells treated with oleate [0.25 mM] plus or minus selenium [100nM] were assayed using a cytometric beads array kit. The experiment shown is a single experiment done in triplicate. * $p<0.05$, ** $p<0.01$ compared to corresponding control (selenium-deficient).

6.3.1.2.3 The effect of co-culture of C3A and LX-2 cells on the LX-2 α-1 pro-collagen mRNA expression

Because an effect of conditioned medium was not clearly observed in the above experiments, it was necessary to conduct an experiment involving co-culture of LX-2 cells with C3A cells that had previously been either fat-loaded, treated with selenium, t-BuOOH or IL-8. For this purpose, the experiment was conducted according to protocol 6.2.4.

As shown in figure 6.5A, only LX-2 cells co-cultured with C3A cells pre-treated with either selenium plus oleate or selenium plus t-BuOOH showed a small but significant difference compared to control (LX-2 cells not co-cultured). Co-cultured selenium deficient C3A cells treated with oleate or t-BuOOH had no significant effect on LX-2 α-1 pro-collagen mRNA expression.

As shown in figure 6.5B, IL-8 treatment of LX-2 cells had no effect on α-1 pro-collagen mRNA expression irrespective of cell pre-treatment with LPON.

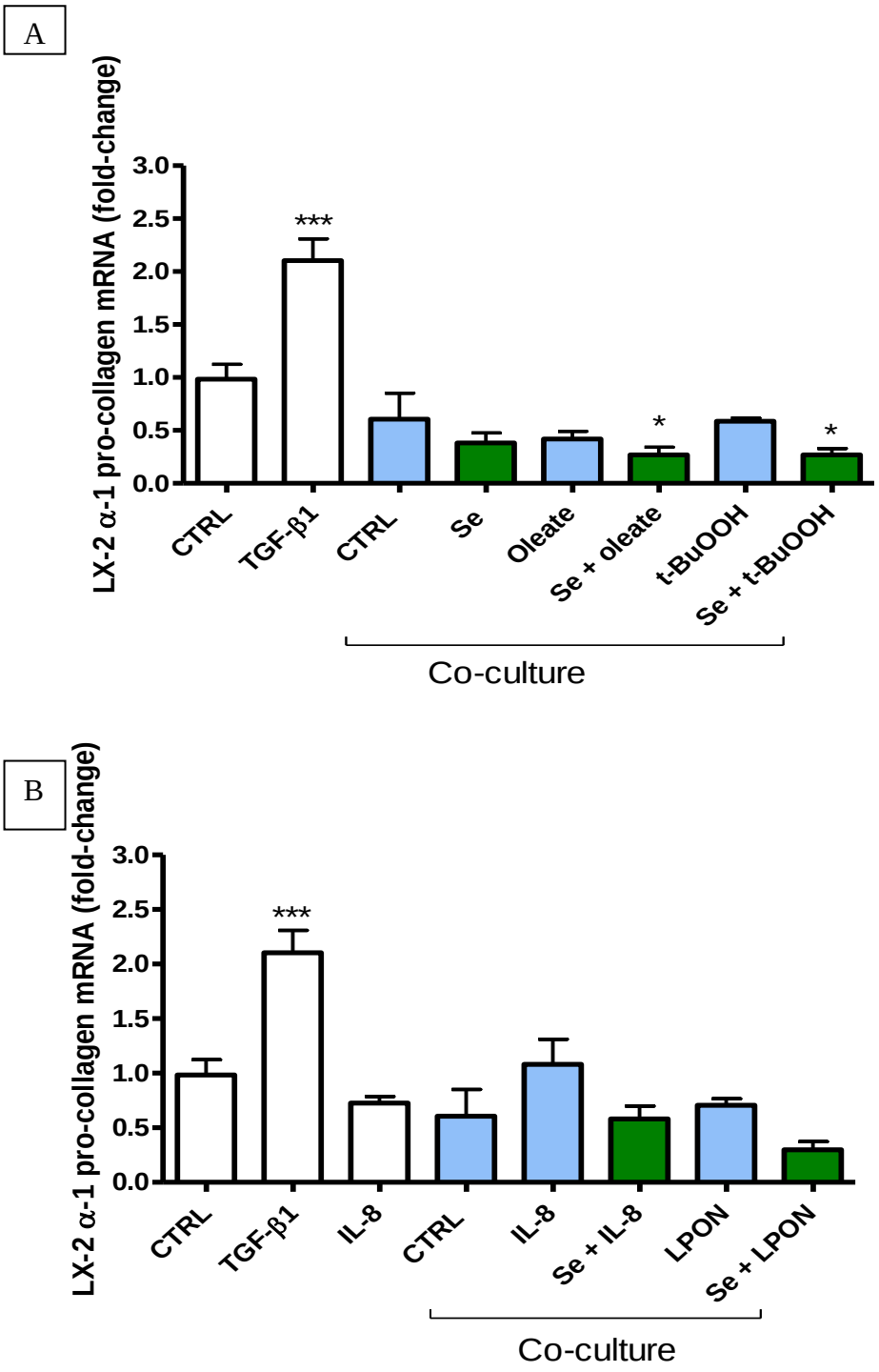


Figure 6.5: The effect of co-culture of variously treated C3A cells on the LX-2 cells mRNA expression of α-1 pro-collagen. C3A cells were treated with oleate [0.25 mM] or t-BuOOH [200 µM] (panel A), IL-8 [500pg/ml] or LPON (panel B) plus or minus selenium [100 nM] for 72 hours. C3A cells were then washed twice and cell culture inserts were then applied to LX-2 cells which had been treated with TGF-β1 [2.5 ng/ml]) and also IL-8 [500 pg/ml] for 24 hours. Data represent the mean ± standard error (± SEM) for three experiments (n=3). Triplicate wells were used for each experiment.* $p<0.05$, *** $p<0.001$ compared to control (CTRL, non-co-cultured cells).

6.3.2 Expression of α-1 pro-collagen mRNA in C3A cells

Because the hepatocyte may also be involved in the process of fibrogenesis in the healing and regeneration process, α-1 pro-collagen mRNA expression in C3A cells was also investigated.

6.3.2.1 The effect of selenium on the TGF-β1-induced α-1 pro-collagen mRNA in C3A cells

Preliminary experiments were carried out to determine the responsiveness of C3A cells to TGF-β1. It was found that 24 hours of incubation was sufficient to induce α-1 pro-collagen mRNA expression in C3A cells (see appendix A6.2.3).

Because C3A cells appeared highly responsive with respect to α-1 pro-collagen expression upon treatment with TGF-β1, the effect of selenium status on the expression of α-1 pro-collagen mRNA in C3A cells was also investigated according to protocol 6.2.5.

As shown in figure 6.6, selenium supplementation was very effective ($p<0.001$) in reducing the expression of α-1 pro-collagen mRNA in response to TGF-β 1. This effect of selenium was seen in non-fat-loaded and oleate-loaded C3A cells such that. α-1 pro-collagen mRNA in selenium-treated cells was approximately only 25% of that found in selenium-deficient cells. Although there was a trend for selenium to reduce the expression of α-1 pro-collagen mRNA in LPON-loaded C3A cells, this did not reach statistical significance.

Following TGF-β1 treatment, LPON plus TGF-β1-cotreated C3A cells had a significantly reduced level of α-1 pro-collagen mRNA compared to TGF-β1 treated cells that were not fat loaded ($p<0.001$). The level of α-1 pro-collagen mRNA in C3A cells was enhanced in oleate loaded cells compared to non-fat loaded cells ($p<0.001$). Treatment with IL-8 had no effect on the expression of α-1 pro-collagen (data not shown).

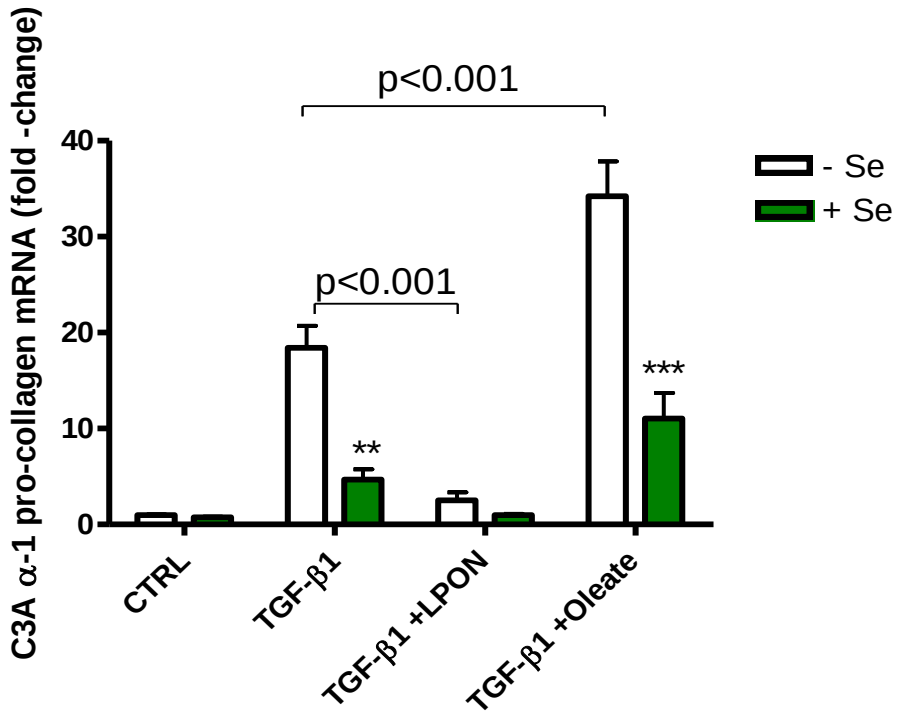


Figure 6.6: The effect of selenium status on the expression of α-1 pro-collagen in C3A cells. C3A cells were divided into two groups, one being selenium-deficient and the second being selenium-replete. After 72 hours, both groups were treated with LPON or oleate [0.25mM] for 48 hours. The C3A cells were then treated with TGF-β1 [2.5 ng/ml], LPON plus TGF-β1 [2.5 ng/ml] or oleate [0.25 mM] plus TGF-β1 [2.5 ng/ml] for 24 hours. The cells were then collected and assessed for α-1 pro-collagen mRNA as described in section 2.2.10. Data represent the mean ± standard error (± SEM) of three experiments n=3, each experiment done using triplicate wells. ** $p<0.01$, *** $p<0.001$ compared to selenium-deficient non-fat-loaded cells (CTRL).

6.4 Conclusion and Discussion

Conclusion:
In conclusion, my research revealed several important points:

1. Neither direct oxidative stress nor indirect oxidative stress (through conditioned medium and co-culture) could promote α-1 pro-collagen expression in partially activated hepatic LX-2 stellate cells. Indeed, paradoxically, induction of oxidative stress via t-BuOOH or LPON treatment appeared to diminish the production of α-1 pro-collagen mRNA. LX-2 cells are known to be a partially activated cell and may be more than 80% activated in normal culture. Thus this cell model may have little reserve for any further induction of α-1 pro-collagen mRNA production in response to stimulation with a range of activators of fibrogenesis. LX-2 cells might not, therefore, be a good model to dissect the pathophysiology of α-1 pro-collagen expression in hepatic stellate cells unless a method is found to achieve a more quiescent state for these cells.

2. LX-2 cells were responsive to an indirect effect of selenium through the use of conditioned medium and co-culture with C3A cells. This effect however was very small and is unlikely to represent an important phenomenon *in vivo*.

3. In C3A cells, α-1 pro-collagen expression could be induced some 10–fold in response to TGF-β1 treatment reaching levels of expression achieved in fully activated LX2 cells.

4. Selenium supplementation was able to markedly reduce the expression of the α-1 pro-collagen mRNA in C3A cells in response to TGF-β1 treatment.

Discussion

In our study, selenium status had no significant effect on α-1 pro-collagen expression by LX-2 cells. This would be consistent with the possibility that the LX-2 cells are already highly activated and could not be de-activated by reducing the levels of oxidative stress following selenium supplementation. Surprisingly the use of t-BuOOH to induce an oxidative stress down-regulated α-1 pro-collagen mRNA production. A possible explanation for this was that the cells' viability was significantly compromised in the presence of t-BuOOH such that the cells were no longer able to synthesise proteins for export (Figure 6.2).

We then investigated if selenium could indirectly exert any effect on α-1 pro-collagen mRNA production by LX-2 cells through modification of the paracrine actions of C3A cells. To investigate this phenomenon, conditioned media from control, fat-loaded, oxidatively stressed, or selenium-protected C3A cells were applied to LX-2 cells to mimic an *in vivo* paracrine effect. In this system selenium treatment of C3A cells had only a comparatively small effect on α-1 pro-collagen mRNA expression, in that selenium diminished α-1 pro-collagen mRNA levels by only 25% (figure 6.3).

The cytokine contents of these conditioned media from C3A cells were also measured (figure 6.4). Although a number of candidate cytokines were assayed only IL-8 was present at detectable levels in these conditioned media. IL-8 is a neutrophil chemoattractant involved in provoking the inflammatory process (Joshi-Barve et al., 2007). Selenium pre-treatment of C3A cells decreased the level of IL-8 of in non-fat-loaded and oleate-loaded cells. IL-8 levels were unaltered by LPON or t-BuOOH treatments. Oleate pre-treated C3A cell produced higher levels of IL-8 compared to control cells. These data might suggest that hepatocytes fat-loaded with oleate are vulnerable to injury and the release of an inflammatory cytokine which would then signal wound repair.

Because certain signals involved in cell to cell communication might be labile or transient, experiments involving co-culture of C3A and LX-2 cells were performed

to investigate further any possible paracrine actions of C3A cells on LX2 cells. These co-culture experiments showed that selenium pre-treatment of C3A cells gave a 50% inhibition of the expression of α-1 pro-collagen mRNA in LX-2 cells (Figure 6.5). Although this effect of selenium on C3A cells was relatively small, this might suggest that hepatocytes, when well-protected from oxidative stress through optimal expression of selenoenzymes, may either fail to release paracrine factors that stimulate α-1 pro-collagen mRNA in LX-2 cells or alternatively selenium-loaded cells may secrete paracrine factors that prevent the synthesis of collagen by stellate cells.

Although there was a trend for an increased expression of α-1 pro-collagen in LX-2 cells treated with conditioned medium from C3A cells exposed to IL-8, such differences were not statistically significant. However, this might suggest that when C3A cells were pre-treated with IL-8, (a known neutrophil chemoattractant and inflammatory cytokine) they would release further chemical signals to hepatic stellate cells to increase the deposition of collagen. It seems reasonable to hypothesise that in cases of inflammation, different signals are released from the hepatocytes to induce inflammation and fibrosis. Since IL-8 had no direct effect on LX-2 cells this might suggest that IL-8 is not directly involved in the regulation of the α-1 pro-collagen but rather connected through inducing hepatocyte inflammation.

There is growing evidence that TGF- β1 is the principal cytokine involved in the activation of stellate cells (Sato et al., 2003). In our LX-2 cell model these cells were already significantly activated and thus exposure of these cells to TGF-β1 could only up-regulate α-1 pro-collagen mRNA two-fold. Indeed, judging from comparison of respective real-time PCR cycle times (C_T values) of C3A and LX-2 cells, the latter could be considered almost fully activated. C3A cells (hepatocytes) were also investigated since these cells may represent the epithelial origin of stellate cells. The expression of α-1 pro-collagen in the C3A cells was very low (C_T values) but when treated with TGF-β1, the expression was increased markedly reaching levels similar to that found in LX-2 cells (Figure 6.6). These observations suggest that these epithelial hepatocytes can be activated and express α-1 pro-collagen to the level of a

highly activated hepatic stellate cell. It was of interest that oleate-loaded C3A cells gave enhanced production of α-1 pro-collagen mRNA when compared with control cells. If hepatocytes contribute to the pathogenesis of fibrosis, our observations are consistent with the view that fat-loaded hepatocytes are more fibrogenic than non-fat-loaded hepatocytes. In contrast to the use of oleate, cells that were fat-loaded with LPON showed a diminished production of α-1 pro-collagen mRNA in response to TGF-β1. This highlights the importance of using an appropriate *in vitro* cell model for NAFLD. On the other hand, it is also possible that the hepatocytes produce collagen as part of the regeneration process to allow anchoring of new cells and eventual repair and not necessarily contribute significantly to the pool of hepatic stellate cells or liver fibrosis.

In our study selenium supplementation significantly suppressed the expression of α-1 pro-collagen mRNA in C3A cells in either the basal state, after stimulation with TGF-β1 or when fat-loaded with oleate. This effect of selenium is likely to be due to the induction of selenoenzymes including TR1 and GPX1 that have antioxidant action (Chapter three). This might suggest that these cells, when supplemented with selenium, become protected from oxidative stress and in turn prevents the activation of the pathways involved in α-1 pro-collagen production in the hepatocyte.

These data provide evidence that selenium is an important protective factor for both oxidative stress and fibrogenesis in normal or fat-loaded C3A cells when exposed to a potent fibrogenic agent like TGF-β1. However, in the case of LX-2 this effect of selenium is difficult to observe given the fact that LX2 cells are already largely activated. Therefore, a quiescent phenotype of LX-2 cells might be a better model to investigate the factors that modify stellate cell production of α-1 pro-collagen gene expression in early stages of liver fibrosis. Selenium pre-treated cells may therefore be better protected from oxidative damage and fibrogenesis and limit the progress of liver fibrosis. The extent to which selenium contributes to reducing the fibrotic response to fat-loading of the liver *in vivo* merits further investigation, as well as the timescale of this protection (i.e. whether protection is early or late, or both, in the fibrotic process). With some agents that protect against fibrosis the protection

afforded may be limited to the earlier stages of fibrosis. An example of this is phosphatidylcholine which is only beneficial in the early stages of fibrosis and has no effect on later stages of the disease (Mato et al., 1999).

In conclusion, this chapter highlights the potential ability of selenium to limit hepatocyte collagen production following exposure to TGF-β1. Given that hepatocytes are ten times more prevalent in the liver than stellate cells the selenium status of the hepatocyte may well influence the pathogenesis of fibrosis in NAFLD in an important way that is amenable to further clinical evaluation.

7 CHAPTER SEVEN

ASSESSMENT OF SELENIUM AND SELENOENZYMES IN OBESITY AND IN PATIENTS WITH TYPE 2 DIABETES

7.1 Introduction

Selenium is an essential trace element for human health through the expression of a wide and growing range of selenoproteins which have important anti-oxidant roles. The current recommended daily allowance (RDA) for selenium is 75 µg in males and 60 µg in females (Rayman, 2000). The highest intake of selenium that is regarded as being non-toxic is 400µg/day (Navarro-Alarcon and Lopez-Martinez, 2000). In subjects ingesting selenium in their diets at doses below the RDA, a strong correlation between the plasma selenium and the GPX1 expressed in red blood cells is found (Koller et al., 1984). Indeed the RDA for selenium is derived from the daily intake of selenium which is required to maximally express GPX1 in red blood cells. The plasma selenium concentration needed to optimize the expression of red cell GPX is approximately 95 µg/l (range 89–114 µg/l) (Rayman, 2000).

The current average intake of selenium in the UK is in the order of ~30 µg/day (Rayman, 1997). The selenium intake in the UK and many parts of Europe has fallen over the last thirty years as a consequence of changes in the source of wheat consumed in many European countries (Rayman, 2000). Most of the dietary selenium consumed in Europe including the UK is in the form of bread and other wheat products. Prior to 1970 much of the wheat used in Europe was imported from areas of North America that had selenium-rich soil. As a consequence, wheat grown in such areas had a high selenium content. Because of this, the average daily intake of selenium at this time was in the order of 65 µg/day. However, after members of the European Community were encouraged to buy goods from within the Common

Market (members of the European community), much of the wheat consumed by Europeans had low selenium content because it was grown in areas where the selenium content of the soil was low. Currently the average selenium intake in the United Kingdom and many parts of Europe is thought to remain at only approximately 40% - 50% of the RDA.

Many selenoproteins are enzymes that have antioxidant actions, and contain selenium in the form of selenocysteine at the active site. These selenoenzymes are involved in redox reactions and are capable of eliminating reactive oxygen species (ROS) and products formed during normal respiration. Two of the most important families of selenoproteins in this regard are the glutathione peroxidases (GPX) and thioredoxin reductases (TR).

Because our hypothesis is that the pathogenesis of NAFLD involves insulin resistance and unopposed oxidative stress, the assessments of selenium and selenoenzyme status in patients at high risk of developing NAFLD may provide indirect evidence that selenium availability may affect the ability to resist oxidative stress and aid the development of NAFLD.

Because obesity and type 2 diabetes are known to be risk factors for developing NAFLD we investigated if the selenium status of healthy subjects and patients with type 2 diabetes was associated with age, BMI, and the waist to hip ratio.

The aims of this chapter are:

1. To assess the selenium status of patients with type 2 diabetes (with or without NAFLD) as determined by plasma selenium concentrations and red cell GPX1 and TR1 expression.
2. To compare these findings with a healthy control group.

7.2 Methods

Study Groups

Patient group

Patients attending the diabetic outpatient department of the Royal Infirmary of Edinburgh were considered for inclusion in the study.

Patients who had any of the following criteria were excluded.

- Alcohol consumption of more than 21 units weekly
- Taking any medications that known to cause fatty liver or deranged liver function tests (LFTs)
- Having other causes of fatty liver or abnormal LFT
- Any co-existing major illness, e.g., malignancy

Blood was collected from these 49 patients and serum, EDTA plasma and red cells were stored at -80^0C until analysed. Normal liver function tests (bilirubin, ALT, alkaline phosphatase, gamma glutamyl transferase, albumin) were found in 30 of these patients although two of these patients had fatty change of the liver demonstrated by the ultrasonography. In the other 19 diabetic patients deranged LFTs were found.

Healthy Control Subjects

Samples were obtained from 133 healthy subjects from two studies. 44 subjects were taking part in the SELGEN study organised by the University of Newcastle (Meplan et al., 2007). The original purpose of this study was to assess whether genetic polymorphisms in the selenoprotein P gene had an effect on selenoprotein expression. These samples were collected from healthy volunteers in Newcastle, UK aged between 20-60 years old who did not have any cardiovascular, hepatic, gastrointestinal or thyroid disease. Also these volunteers were non-smokers, had an alcohol intake of less than 30 units/week, were not taking any selenium or multivitamin supplements nor on any anti-inflammatory medication (Meplan et al., 2007). In addition, the healthy control group comprised 89 subjects from Aberdeen who were enrolled for a study of the effect of various diets on blood pressure. All

control subjects had been taking their "normal" diet prior to being enlisted and ethical approval was obtained for all studies

Methods

Plasma Selenium

Selenium levels were kindly measured by Mr Gordon Marr (Combined Laboratories, Royal Infirmary of Edinburgh) using atomic absorption spectrophotometry (see section 2.2.11).

All liver function tests (bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin and gamma glutamyl transferase) were measured in serum using an Olympus 2700 analyser in routine use in the Clinical Biochemistry laboratory at the Royal Infirmary of Edinburgh.

The BMI was measured from the height and body weight as follows
BMI= weight (Kg)/height ^{2}(m).

With the co-operation of Professor Hayes group at the Royal Infirmary of Edinburgh, the collection of blood samples was carried out by Dr Ann Lockman from the diabetic out-patient clinic and by Mrs Lesley McGregor from the liver out-patient clinic.

The study had a full ethical approval. Blood samples were then sent to the clinical biochemistry research laboratory where I assessed the TR1 and GPX1 levels. Once received in our research lab, they were frozen and stored at -80^{0}C until analysed. Samples were diluted 350 fold to assess the TR1 as described in section 2.2.8 and diluted 20 fold to assess the GPX1 as described in section 2.2.3.2.

7.3 Results

7.3.1 Plasma selenium concentrations in the healthy group and patients with type 2 diabetes mellitus

It was found that 97.5% of the healthy group had plasma selenium concentrations which were below the recommended optimal selenium concentration (fig 7.1). The recommended plasma selenium level to maximally express GPX1 is about 95 µg/l (range 89-114 µg/l) (Rayman, 2000). These observations are consistent with other evidence that indicates the selenium status in the UK is well below the levels needed for optimal GPX1 expression in healthy populations. Mean selenium was (63.73 ± 0.99 µg/l) of all the recruited volunteers.

The mean plasma selenium concentration in patients with type 2 diabetes mellitus was 58.81 ± 2.0 µg/l, N=49 which was significantly lower than the concentrations found in the healthy subjects 63.73 ± 0.99 µg/l, N=133 (figure 7.1), ($p<0.01$) (Mann Whitney test). The range of selenium concentrations found in the patients was also wider than seen in the healthy subjects.

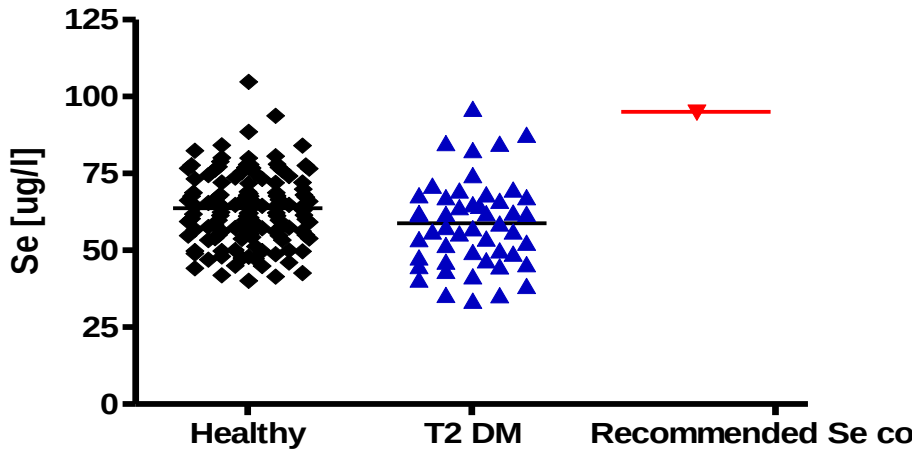


Figure 7.1: The plasma selenium levels in diabetes mellitus patients in comparison with healthy subjects ($p<0.017$). The red line represents the recommended selenium concentration to maximally express the GPX1 in red cells. Healthy group, n=133; diabetic group, n=49.

7.3.2 Correlation of plasma selenium, red cell TR1 and red cell GPX1 in the healthy and type 2 diabetes mellitus patients

Selenium

The selenium concentrations were studied further to see if there were any associations with the various demographic and measured parameters which included:

1. Age of the patient
2. The duration of the diabetes mellitus
3. Weight
4. Waist to hip ratio
5. Blood pressure
6. Alanine aminotransferase (ALT)
7. Aspartate aminotransferase (AST)
8. Alkaline phosphatase (ALP)
9. AST/ALT ratio
10. AST/platelets
11. Gamma glutamyl transferase (GGT)
12. Bilirubin
13. Albumin
14. Fasting glucose
15. HbA1c
16. Total cholesterol
17. Triglyceride level
18. High density lipoprotein (HDL)
19. Low density lipoprotein (LDL)

There was no correlation between selenium levels and any of these parameters with the exception of BMI.

As shown in figure 7.2A and B, there was a significant negative correlation (Pearson correlation) between selenium levels and Body Mass Index (BMI) of the healthy group (A) ($p<0.0001$; Pearson constant (r) =-0.28) and diabetic group (B) ($p<0.005$;

Pearson constant (r) = -0.39). However, there was no correlation between waist to hip ratio and the level of selenium in the diabetic group (data not shown, p= 0.1923). Waist-hip ratio data were not available for the control group.

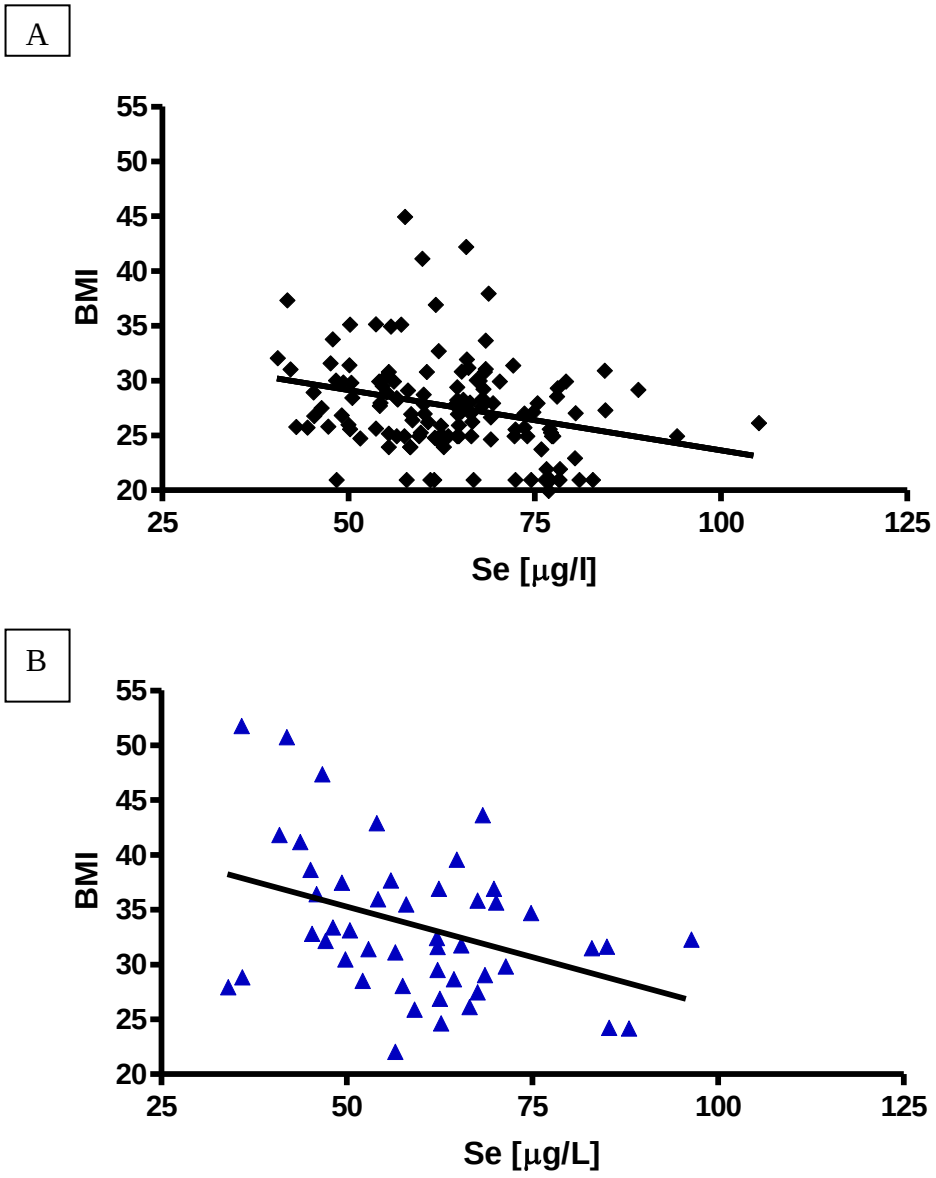


Figure 7.2: Negative correlation between BMI and plasma selenium in (A) healthy group and (B) Type 2 diabetes mellitus patients.

Correlation of plasma selenium with functional markers of selenium status, red cell GPX1 and TR1 in patients with type 2 diabetes mellitus

A significant correlation was found between GPX1 activity in red blood cells and plasma selenium levels (Pearson correlation, r = 0.34) (Figure 7.2 A). However, there was no significant correlation (Pearson) between plasma selenium and red blood cell TR1 level (p= 0.40) (figure 7.2 B)

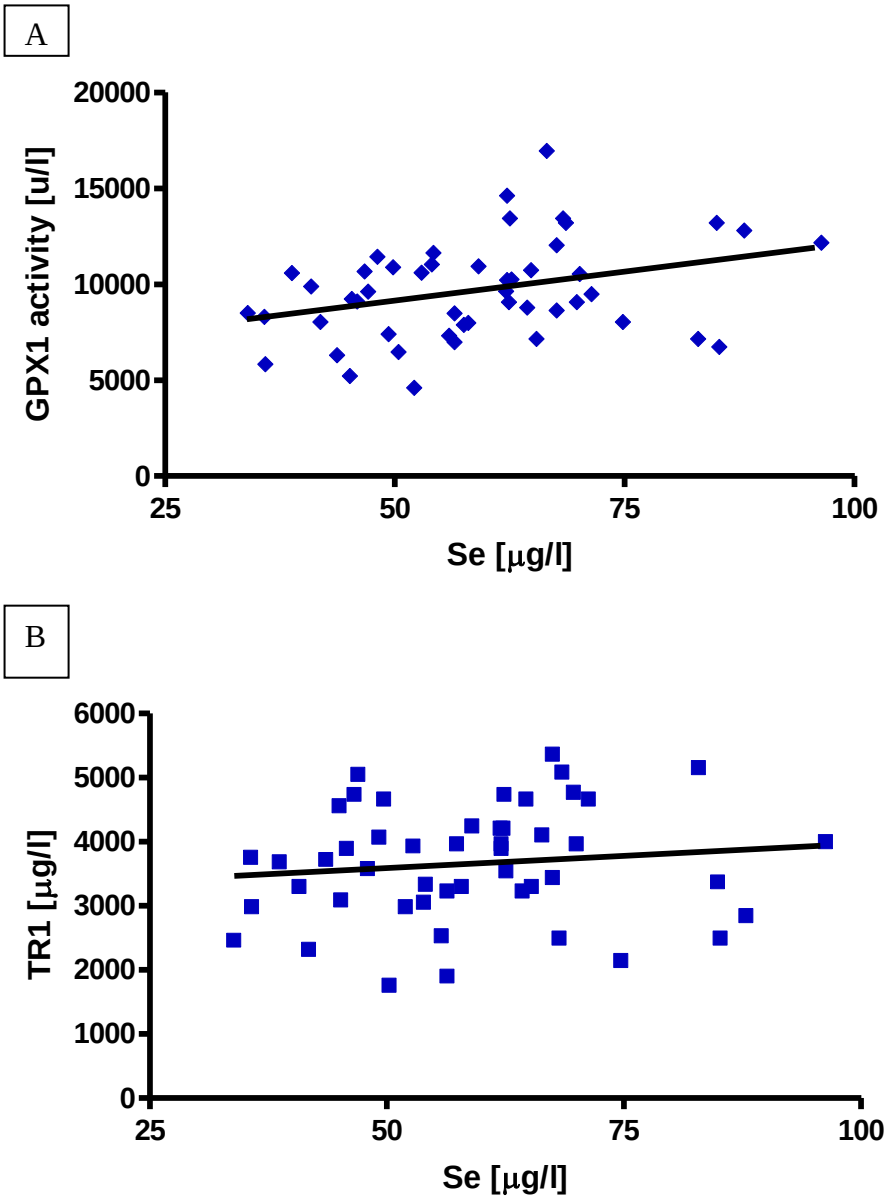


Figure 7.3: Correlation of red cell GPX1 activity (A) and red cell TR1 concentration (B) with plasma selenium concentrations in patients with type 2 diabetes mellitus. p<0.02 (A) and p=0.40 (B).

Comparison of selenium status in type 2 diabetes patients with normal LFTs with those patients exhibiting abnormal liver function.

Selenium status was compared in patient with abnormal liver function tests compared to those whom liver function tests were normal.

As shown in figure 7.4A, there was no significant difference between plasma selenium in patients with normal LFTs (59.55 ± 2.67 µg/l, N=30) compared with patients exhibiting abnormal liver function (57.64 ± 3.1 µg/l, N=19). There were no significant differences between red cell GPX1 in patients with normal LFTs (9452 ± 45 µg/l, N=30) compared with patients that had abnormal liver function (10040 ± 60 µg/l, N=19) (figure 7.4 B). Similarly TR1 levels were no significantly different between the two groups. TR1 levels in normal LFTs group were (3787 ± 15 µg/l, N=30) versus abnormal LFTs group (3456 ± 22 µg/l, N=19) as shown in figure 7.4 C.

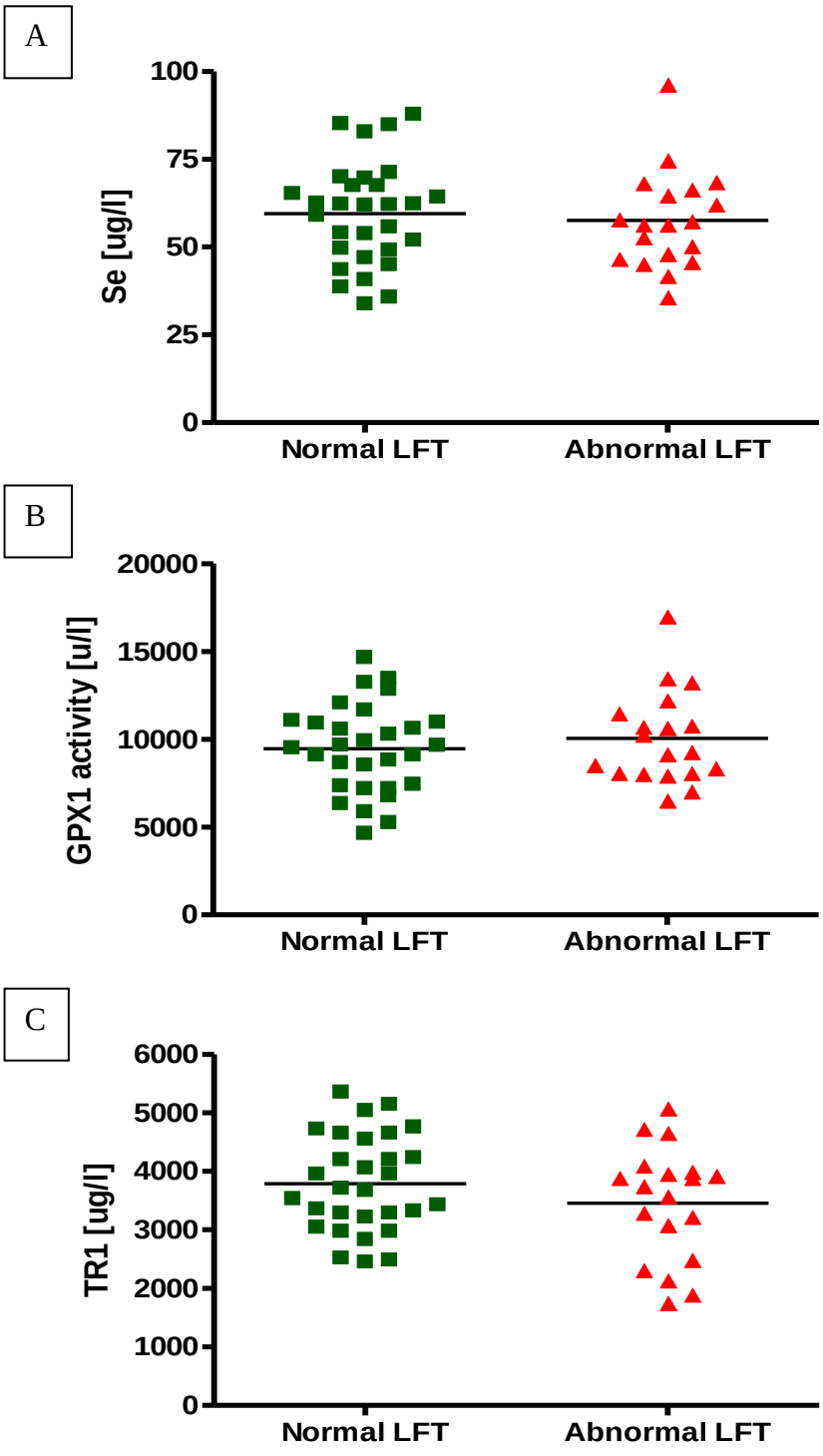


Figure 7.4: Comparison of selenium GPX and TR1 levels between normal and abnormal LFTs groups of type 2 diabetes mellitus. (A) Selenium level, (B) GPX1 level and (C) TR1.

7.4 Conclusion and Discussion

Conclusion:

In this chapter I have presented data that show obesity leads to a diminution in functional selenium status in both plasma and at the tissue level (red blood cells). Obesity is a strong risk factor for the development of both insulin resistance and NAFLD. Thus the data are consistent with the following hypothesis which has been the theme throughout this thesis: namely, obesity leads to insulin resistance resulting in mobilisation of fatty acids to the liver where they exert an oxidative stress through β-oxidation in the mitochondria. Obesity also leads to a diminution in antioxidants including selenoenzymes. This in turn leads to unopposed oxidative stress with resultant oxidative damage and release of inflammatory cytokines and other chemokines. These chemokines promote steatohepatitis that activates hepatic stellate cells and leads to collagen production and fibrosis.

This plausible hypothesis outlines the need for selenium supplementation intervention trials in human obese subjects to determine if such an intervention can prevent such patients from developing NAFLD. However the doses of selenium used for such a trial should be chosen with caution since there is some evidence to suggest that supplementing with 200 micrograms of selenium per day increases the risk of developing type 2 diabetes (Bleys et al., 2007).

Discussion

In our study, both the healthy subjects and patients with type 2 diabetes mellitus had plasma selenium levels that were considerably below that required to optimally express glutathione peroxidase (GPX) activity in red blood cells (range 89-114 μg/l) (Rayman, 2000). This highlights the desirability of selenium supplementation even in healthy UK residents. These findings on healthy control subjects confirmed previous studies in the UK, which suggest that selenium intake is only 40-50% of the recommended daily allowance (Table 7.1, Rayman, 2000).

In this chapter we have shown that patients with type 2 diabetics have significantly lower plasma selenium concentrations as compared to a healthy population. Furthermore, type 2 diabetics also have decreased stores of other anti-oxidants including GPX1 (Vericel et al., 2004). This would imply that this group of patients is more prone to the effect of oxidative stress (the second hit) and that they may be at an increased risk of developing NAFLD and particularly its progression from fatty liver to NASH and cirrhosis.

The question arises as to whether type 2 diabetes leads to a lower selenium status or whether a deficiency in selenium status occurs before the onset of the disease. The control population used in this study were derived from the SELGEN study (Meplan et al., 2007) and from subjects who were enrolled for a study of the effect of various diets on blood pressure in Aberdeen. In these healthy subjects and in the patients with diabetes, a significant inverse correlation was found between BMI and plasma selenium. Obesity per se thus appears to lead to a diminution in selenium status prior to the onset of type 2 diabetes.

In addition, the plasma selenium concentrations in the diabetic patients correlated significantly with their red cell GPX activities. This supports the possibility that the low plasma selenium concentrations lead to a functional selenium deficiency at the tissue level rather than the low plasma selenium being a result of changes in plasma selenium binding proteins and an acute phase reaction (Behne et al., 1996).

Why selenium status is inversely correlated with BMI is unclear. Food intake in the obese would be high but if calories were predominantly taken as fat then this would not provide a suitable dietary source of selenium. Interestingly the use of the waist to hip ratio as a measure of obesity showed no inverse correlation with plasma selenium. The BMI represents the total body fat in contrast to the waist to hip ratio which reflects only the abdominal or visceral fat. The inverse correlation of BMI with selenium levels suggests that total body fat of type 2 diabetics is a more important predictor of low selenium status than the waist to hip ratio in.

There was no correlation between TR1 and selenium levels in the type 2 diabetic patients despite a positive correlation between GPX1 and selenium being found. This is consistent with the hierarchical model of selenoprotein expression where, in selenium deficiency, GPX is the most sensitive of the selenoproteins to loss of expression in selenium deficiency (Hill et al., 1997a). TR falls above GPX in this hierarchy as demonstrated in Chapter three. Thus with the degree of selenium deficiency found in the UK, it appears TR1 expression can be maintained at maximal levels but GPX expression falls significantly below the maximum possible.

There were no differences in plasma selenium, red cell GPX1 or red cell TR1 levels between diabetic patients with normal LFTs and those with abnormal LFTs (indicating active pathology of the liver). This observation might arise because LFTs do not accurately reflect the severity of liver pathology given that patients with various stages of NAFLD may have normal ALT (Mofrad et al., 2003).

In conclusion, in this chapter I have presented data that show obesity, a strong risk factor for the development of NAFLD, leads to a diminution in functional selenium status. If oxidative stress is involved in the pathogenesis of NAFLD it seems plausible that obesity and low selenium intake in the UK are risk factors for development and progression of NAFLD. This plausible hypothesis outlines the need for further studies and possibly a selenium supplementation intervention trial to be performed in obese subjects to determine if such an intervention can prevent such patients from developing NAFLD. However the doses of selenium used for such a trial should be chosen with caution since there is now evidence that supplementing with 200 micrograms of selenium per day increases the risk of developing type 2 diabetes (Bleys et al., 2007).

8 CHAPTER EIGHT

FINAL DISCUSSION AND FUTURE STUDIES

8.1 Introduction

The aim of this thesis was to test the hypothesis that selenium-deficiency is an important contributor to the pathogenesis of NAFLD and progression to NASH and fibrosis. The rationale for this hypothesis is that an inadequate dietary selenium supply (as occurs in the UK population) would lead to poor expression of selenoenzymes that have important antioxidant actions. This, in turn, would promote unopposed oxidative stress arising from fatty acid metabolism in the mitochondria of fat loaded hepatocytes and hepatic stellate cells. Subsequent oxidative damage to the hepatocyte and hepatic stellate cell would produce an inflammatory response that would activate the stellate cell resulting in the production of fibrogenic proteins.

In order to test this hypothesis it was important to develop fat-loaded cell models that were viable in selenium-deficient culture medium. Such cells could then be used to assess the effect of selenium supplementation on fatty acid and lipid hydroperoxide toxicity, α-1 pro-collagen expression and selenoenzyme expression.

Development of selenium-deficient cultured hepatic cell models (Chapter three)

This thesis has established that both C3A and LX-2 cells, representative of human hepatocytes and hepatic stellate cells respectively, are capable of expressing key selenoenzymes whose expression can be modulated by selenium supply. In chapter three, it was revealed that IT-supplemented medium was a suitable selenium deficient medium which allowed good cell viability and subsequent induction of selenoenzymes following selenium supplementation.

To characterize which components of the IT-medium were required for maintenance of cell viability the individual components of the IT medium (i.e. insulin and transferrin) were evaluated. Transferrin (as a bulk protein) added alone failed to improve cell viability in both cell types, even at high concentrations. This suggests that C3A and LX-2 cells need more than simply added bulk protein for adequate viability. In contrast, insulin was clearly the factor in the IT-supplement which was required to maintain cell viability. It is thought that insulin exerts a mitogenic effects on the cells through the expression of the insulin-like growth factor receptor, IGF-1 receptor (Polychronakos et al., 1991). ITS-supplement is commonly used in cell culture to provide an ideal serum-free medium since it contains the iron-carrying protein (transferrin), growth factor (insulin) and the anti-oxidant factor (selenium) (Kisiday et al., 2005). By removing selenium from the ITS supplement we achieved cell viability under selenium-free conditions using IT medium alone.

It is likely that, FBS-supplemented medium contains insulin-like growth factors , which provide this growth-promoting function (Giacca et al., 1994) but, as shown in chapter three, FBS contains selenium that is accessible by some cell types. This makes FBS-containing medium unsuitable for studying the effects of selenium deficiency.

Insulin and transferrin added either singly or in combination, had no effect on the expression of TR1. This allowed IT-medium to be used to monitor selenoenzyme expression under different incubation conditions, including fat loading or selenium supplementation. In particular, selenium supplementation of these cells grown in IT-medium (by addition of selenite to the medium) resulted in an increased expression of the selenoenzymes TR1 and GPX1 when compared with cells grown in insulin and transferrin alone (selenium-deficient cells). As these selenoenzymes are considered to be important intracellular anti-oxidants, this allowed me to pursue the central hypothesis of this thesis using these cell model systems in states of selenium depletion or repletion.

Models of fat-loading using different fatty acids (Chapter four)

Fat accumulation in the liver per se is considered by some to be physiological and does not necessarily occur following a hepatotoxic insult such as long-term excess alcohol intake. In some species, such as migratory birds, fat accumulation in the liver is considered a physiological process to help them undertake long journeys using the accumulated fat as the main source of energy (Bairlein, 2002; Jansen, 2004). Furthermore, a recent study suggests that fat accumulation inside the hepatocyte could serve as protective mechanism preventing liver damage following exposure to pro-oxidants and inflammatory cytokines (Damelin et al., 2007). One mechanism by which fat becomes hepato-protective is by directing fatty acids into triglycerides thereby preventing free fatty acids becoming available for oxidation by the mitochondria; this in turn diminishes the accumulation of secondary lipid peroxidation products. In contrast, other reports consider fat accumulation in the hepatocyte as injurious and impairing the normal function of the hepatocytes (Day, 2006).

The complex relationship between triglyceride accumulation and cell injury is highlighted in a recent study using CHO cells. This study showed that triglyceride accumulation in these cells per-se was neither toxic nor made the cells more susceptible to injury (Listenberger et al., 2003) . Furthermore, oleate-treated cells accumulated triglyceride while palmitate-treated cells showed no accumulation of triglyceride. A combination of oleate and palmitate treatment showed some triglyceride accumulation. Oleate might therefore facilitate triglyceride accumulation, thereby protecting the cells from the apoptotic effect of palmitate (Listenberger et al., 2003). It could be therefore argued that these cells accumulate triglyceride as a protective mechanism to minimise injury. On the other hand, it is possible that after longer-term exposure to fatty acids, the cells have no further capacity to safely accumulate triglyceride and subsequent hydrolysis of the accumulated triglyceride occurs resulting in oxidation of the released fatty acids which cause cell injury due to oxidative stress (Listenberger et al., 2003).

The more obese a patient is, the greater are the amounts of fatty acids that can be mobilized from adipose tissue to the liver following the development of peripheral insulin resistance. This large influx of fatty acids can potentially place a huge oxidative load on the hepatic mitochondria where the fatty acids undergo β-oxidation. A secondary consequence of this increased oxidative metabolism (oxidative stress) in the mitochondria is the production of high levels of damaging lipid peroxides. The huge flux of fatty acids to the liver makes it difficult for the hepatocytes to function in a balanced manner and much of this fatty acid entering the hepatocyte then becomes esterified and accumulates as stored triglyceride (fat) in the cytoplasm. Although insulin resistance had been extensively studied, the mechanism of insulin resistance is not a straight forward process and may involve genetic polymorphisms and environmental factors that may promote obesity and other related diseases (Adams et al., 2005; Ong and Dunger, 2002).

It is important to recognize that the oxidation and metabolism of different fatty acids are related to their carbon chain length and degree of unsaturation (Pai and Yeh, 1996). Octanoate, as a medium chain fatty acid, can freely enter the hepatic mitochondria without need of the carnitine transport system. Once in the mitochondria octanoate undergoes β-oxidation (Miele et al., 2003). Moreover, medium chain fatty acids such as octanoate are oxidized much faster than longer chain length fatty acids (Greenberger et al., 1966; Leyton et al., 1987). Other evidence is suggestive that short and medium chain fatty acids enhance the uncoupling of oxidation in the mitochondria and subsequently provoke oxidative stress (Hird and Weidemann, 1966).

In contrast to octanoate, the requirement of oleate to enter the mitochondria only via the acyl-carnitine transporter allows a mechanism by which mitochondrial oleate oxidation is controlled. Therefore excess oleate entering the hepatocyte can be diverted to triglyceride rather than being metabolized in the mitochondria through β-oxidation. Palmitate, a C16 saturated fatty acid, appears to be more toxic to the cell than oleate. Palmitate, but not oleate, induces cell death in pancreatic cells (Karaskov

et al., 2006). Furthermore, palmitate induces acute injury in the hepatocyte while oleate-induced cell damage only after chronic exposure (Gomez-Lechon et al., 2007). Other studies also suggest that saturated fatty acids predispose the hepatocytes to oxidative stress damage (Wang et al., 2006).

Collectively, it is evident from various studies that different fatty acids have differing effects on the susceptibility of a cell to oxidative damage. For this reason two models of fat-loading were investigated in chapter four of this thesis, namely cells loaded with octanoate (as LPON) and ones loaded with oleate or palmitate.

Under normal culture conditions C3A cells do not accumulate lipid and thus are not representative of the fat-loaded hepatocytes seen *in vivo* in patients with fatty liver. However, long-term culture of C3A cells in a medium containing a cocktail of lactate, pyruvate, octanoate and ammonium ion (LPON) not only increases the functionality of C3A cells but also results in the accumulation of fat-droplets, as visualised by electron microscopy (Filippi et al., 2004). The work presented in chapter four confirmed the observations of Filippi et al and also showed that the fat droplets were comprised largely of triglyceride and not cholesterol. Both C3A and LX-2 cells were successfully fat-loaded using LPON treatment and fat loading led to increased levels of TR1 and GPX1, expression, consistent with increased oxidative stress, possibly through an un-regulated metabolism of octanoic acid in mitochondria.

The second model of fat-loading employed oleate and palmitate treatments and was based on an earlier study describing the fat-loading of HEPG2 cells (Gomez-Lechon et al., 2007). Fat-loaded C3A and LX-2 cells after oleate or/and palmitate treatments were shown to have higher triglyceride accumulation compared to LPON-treated cells. These cells did not demonstrate increased levels of either TR1 or GPX1 suggesting that this second model of fat-loading did not provoke the same degree of oxidative stress as that found in LPON treated cells. This may possible be due to the regulated entry of long-chain fatty acids into mitochondria. Because, under normal dietary conditions, fatty acids are predominantly ingested as long chain rather than

medium chain fatty acids it could be concluded that oleate-loaded cells provide a more physiologically relevant model.

Since fat-loaded hepatic stellate cells *in-vivo* are believed to be quiescent cells, it was of value to explore the mechanism of stellate cell activation and its relation to fat loss during the process of activation, as this area is unclear.

In chapter four appendix, the ability of LX2 cells to loose their triglyceride in case of activation (addition of TGF-β1) was tested. Although, this mechanism has been proposed as a hypothesis for activation (Friedman et al., 1993), it could not be confirmed in my experiments. This might suggest that the decrease in triglyceride per se is not involved in the activation process but rather that a unique cytokine such as PDGF is required for activation (Friedman, 2003). There is very little information about the role of retinoids and the relationship of retinoid loss to the activation process. Furthermore, LX-2 cells seem to be largely activated cells *in-vitro* (80% activated) and fat loading these already activated cells might not render them quiescent. Indeed, fat loading LX2 cells had no effect on α-1 pro-collagen expression; implying fat loading had no effect on the activation state of these cells.

The effect of selenium status and fat loading on the susceptibility of cultured liver cells to damage by oxidative stress induced by lipid hydroperoxides (Chapter five)

Low plasma selenium levels in humans have been associated with many diseases including cardiovascular and neurodegenerative diseases (Castano et al., 1997; Kok et al., 1985). Selenium deficiency can result in an accumulation of fatty acid peroxides in the liver and lead to the formation of ROS products that enhance oxidative stress. Cells cultured under selenium-deficient conditions accumulate lipid hydroperoxides more rapidly under conditions of oxidative stress than selenium sufficient cells, and die more rapidly (Geiger et al., 1993). Selenium supplementation can prevent oxidative stress and hepatic cell damage induced by

lipopolysaccharide (LPS) treatment (Kim et al., 2004) and also cell damage induced by t-BuOOH treatment of cultured human endothelial cells (Campbell et al., 2007).

The findings presented in chapter five show that selenium was a major protective element against oxidative stress induced by t-BuOOH in both C3A and LX2 cells. Moreover, selenium could provide this protection when C3A and LX2 cells were fat-loaded. Pre-treatment of cells with selenium for 48 hours was required for cells to acquire protection, suggestive that synthesis of selenoenzymes was required for selenium to exert its protective effect.

The GPX and TR selenoenzymes are powerful antioxidant enzymes in the cell cytoplasm (Sies, 1997). Both enzymes can catalyses the reduction of a variety of hydroperoxides including hydrogen peroxide, t-BuOOH, and fatty acid hydroperoxides (Brigelius-Flohe et al., 2003; Rotruck et al., 1973). Several studies have shown that GPX activity is increased in response selenium or to oxidative stress (Ibrahim et al., 1997; Kim et al., 2004) and, as shown in chapter four, LPON and octanoate were able to induce these enzymes, though not oleate.

It was not possible to show that LPON-treated cells were more sensitive to oxidative damage by t-BuOOH (a commonly used *in vitro* inducer of oxidative stress) than non-fat loaded cells. This was because of the high variability between the three experiments in the doses of t-BuOOH required to cause cell damage. However there was a clear trend in each individual experiment to show that LPON-treated cells were more susceptible to t-BuOOH toxicity than non-fat-loaded cells; this was true regardless of the selenium status of the cell. There was no evidence to suggest that oleate treatment had any protective or sensitising effect with regard to damage induced by t-BuOOH. It can be concluded that selenium can provide C3A and LX2 cells with marked protection from oxidative stress induced by t-BuOOH and this occurs irrespective of whether such cells are fat-loaded. Although not conclusive, the data presented in chapter five strongly suggest that octanoate but not oleate induces a marked oxidative stress in these cells and predisposes them to damage by lipid hydroperoxides.

α-1 pro-collagen expression in C3A and LX-2 cells (Chapter six)

Hepatic stellate cells are normally quiescent and store vitamin A. However, in liver injury, they undergo excessive cellular proliferation and transform into a highly contractile cell type known as the myofibroblast. The mechanism of hepatic stellate cell activation, described in several papers, has two stages: the first or initiation step renders the cells responsive to various arrays of cytokines released by nearby inflamed cells and the second or perpetuation stage is characterized by a series of events which lead eventually to matrix and fibrosis accumulation (Friedman, 2003). Different cytokines seems to be responsible for different events which occur during activation (Friedman, 2003). The activated phenotype is associated with a reduction of lipid droplets, loss of stored vitamin A (Friedman et al., 1993), increased proliferation, increased synthesis of collagen and alpha-smooth muscle actin, increased contractility and increased deposition of extracellular matrix components, including collagen, proteoglycans and adhesive glycoproteins (Friedman, 2000). Platelet-derived growth factor (PDGF) is responsible merely for the loss of retinoid and it may be a process unrelated to activation itself (Friedman, 2003). The myofibroblasts migrate to the site of hepatic lesions and represent the principal cells involved in collagen production before the establishment and progression of hepatic fibrosis and cirrhosis.

Several studies suggest that effects of oxidative stress on hepatocytes and inflammatory cells such as Kupffer cells and neutrophils may be the main drivers of stellate cell activation (Giuliana Montosi, 1998). Other studies claim that oxidative stress generated within the stellate cell can activate them and thus promote stellate cells to release collagen, leading to liver fibrosis (Houglum et al., 1997). Most of the studies suggestive that oxidative stress could activate stellate cells directly were carried out *in vivo*.

The regulation of α-1 pro-collagen expression in hepatic stellate cells remains controversial. Some studies suggest that the direct effect of injury and oxidative stress on the hepatic stellate cells is the main regulator of α-1 pro-collagen

expression (Lee et al., 2001). Furthermore, one study claimed that rat hepatic stellate cells transfected with cytochrome P4502E1 (CYP2E1), an obligatory essential producer of reactive oxygen species (ROS), could increase α-1 pro-collagen expression 4-fold compared to cells transfected with empty vector (Nieto et al., 1999). In contrast, other studies indicate that α-1 pro-collagen regulation is related to paracrine effects of oxidatively stressed or injured adjacent cells (Svegliati Baroni et al., 1998). Paracrine inflammatory cytokines released from oxidatively stressed hepatocytes or Kupffer cells might therefore have a major role in the process of the activation of hepatic stellate cells (Reeves and Friedman, 2002).

Although many studies suggest that apoptosis or reversion of cell type is the main fate of hepatic stellate cells in liver regeneration, it is not clear why these cells choose to differentiate one way or the other (Friedman, 2000). Furthermore, the regulation of cell reversion is not fully understood. Some studies attempted to drive the activated hepatic stellate cells to apoptosis (Issa et al., 2001; Wright et al., 2001). Others attempted to reverse the oxidative stress state using anti-oxidants such as vitamin E (Agarwal et al., 2005). Vitamin E was also found in inhibit the activation of hepatic stellate cells (Naziroglu et al., 1999). In contrast, another study on silymarin revealed no effect on the process of hepatic stellate cell activation (Pares et al., 1998).

The question as to whether oxidative stress and resulting oxidative damage in LX-2 or C3A cells might lead to the activation of hepatic stellate cells (i.e., LX-2 cells) was addressed in chapter six. We found that neither t-BuOOH, H_2O_2 nor menadione could induce α-1 pro-collagen expression in LX2 cells. This implied that a direct oxidative stress could not further affect these already activated stellate cells. However surprisingly, we found that t-BuOOH (a known inducer of oxidative stress) suppressed α-1 pro-collagen expression. This may be because t-BuOOH might induce a programmed cell death whereby the cell shuts down most of its gene expression entirely. This view was supported by the suppressive action of t-BuOOH on the expression of other genes such as lysyl oxidase and steroid sulphatase.

As t-BuOOH was clearly inhibiting general gene expression in our LX2 experiments, the indirect effect (through conditioned medium and co-culture experiments) of this agent was therefore also investigated. Intriguingly, the use of conditioned medium or co-culture following t-BuOOH exposure had no effect on α-1 pro-collagen expression consistent with a direct toxic action of t-BuOOH on LX2 cells.

In chapter six, cytokine production by C3A cells under a variety of conditions was studied. Only changes in IL-8 could be observed with the release of this cytokine increased by oleate-loading and diminished by selenium supplementation. Palmitate-loaded human hepatocytes also release increased amounts of IL-8 (Joshi-Barve et al., 2007). The mechanism by which the IL-8 is released is believed to be related to the activation of nuclear factor kappaB (NF-kappaB) and c-Jun N-terminal kinase/activator protein-1 pathways (Joshi-Barve et al., 2007). Other studies also suggest that IL-8 is increased in inflamed alcoholic liver (Kershenobich Stal and Weissbrod, 2003).

From chapter six, it is evident that conditioned medium and co-culture studies failed also to alter α-1 pro-collagen mRNA expression in the LX-2 stellate cell line. Thus neither direct nor indirect oxidative stress was found in our study to induce α-1 pro-collagen mRNA expression in LX-2 cells. We postulate that this most probably arose because the LX-2 cells were already activated to a greater level than first believed (the original cells were described as 'partially' activated).

Various studies have indicated that activated hepatic stellate cells are the main producers of α-1 pro-collagen in liver fibrosis (Sato et al., 2003; Xu et al., 2005). Epithelial to myofibroblast transformation occurs in fibrogensis in various organs including kidney (Yang and Liu, 2002) and lung (Burgess et al., 2005; Willis et al., 2006).Recent studies are also suggestive that this phenomenon could occur in liver fibrosis (Diaz et al., 2008; Zeisberg et al., 2007). The major cell type existing in healthy liver is the hepatocyte but the hepatic stellate cells become more abundant in cases of liver fibrosis (Zeisberg et al., 2007).

When α-1 pro-collagen mRNA expression in C3A was investigated, a remarkable response of α-1 pro-collagen mRNA expression to TGF-β1 treatment was observed. The level of α-1 pro-collagen mRNA expression in TGF-β1-treated C3A cells almost reached the level of α-1 pro-collagen mRNA expressed in un-treated LX-2 cells. This also implied that C3A cells might have the potential to transform into myofibroblast-like cells.

Interestingly, selenium was effective in damping α-1 pro-collagen mRNA expression in TGF-β1-treated C3A cells. This raised the possibility that selenium supplementation could be beneficial in the prevention of the transformation process and suppress α-1 pro-collagen mRNA expression in hepatocyte cells. In turn, this might be translated into a potential reduction in fibrosis associated with inflammatory liver disease. The observations given in chapter six are consistent with the hypothesis that there is an epithelial to mesenchymal transition in hepatocytes (C3A cells) following exposure to TGF-β1.

LPON-treated C3A cells were found to exhibit a much lower level of α-1 pro-collagen expression than oleate-loaded C3A cells when exposed to TGF-β1. Indeed since oleate-treated C3A cells showed a higher level of α-1 pro-collagen mRNA than LPON-treated cells, it might suggest that oleate-loaded C3A cells may be, from this one perspective, a better model of fatty liver.

As shown in chapter five pre-treatment of cells with selenium (to optimize the expression of selenoenzymes) led to better protection from oxidative stress injury compared with cells which were not selenium-supplemented. Although, this seems to be the case for both cell types, the effect of selenium supplementation on α-1 pro-collagen gene expression was more obvious in C3A cells than in LX-2 cells.

Selenium and the UK population (Chapter seven)

There have been a number of reports that highlight the problem of the low selenium status of the UK population with results similar to those found in our study. One

study of selenium levels in a healthy group of subjects recruited in Scotland found a plasma selenium concentration of 61 µg/l in males and 60 µg/l in females (Shortt et al., 1997). In the same report, the authors concluded that only 2.5% of the samples collected had an optimum selenium level that would allow maximal expression of GPX and hence the possibility of greater protection from oxidant damage. These findings have potential significance in respect to cancer incidence and other diseases associated with selenium deficiency. A further report observed a low selenium level of 69 µg/l in British people aged between 4-18 years (Bates et al., 2002). Of relevance are the results from a recent study which found that persistently low selenium is associated with an increased risk of developing heart disease and cancer (Bleys et al., 2008).

In our study, none of the samples had a selenium level above 130µg/l with most of them below falling below 75 µg/l. Furthermore, it is not only the UK population who have a low selenium intake but also those in most of Europe are affected to a greater or lesser extent (Rayman, 2000). The average daily intake of selenium in the UK does not exceed 40 µg/day while the recommended intakes for males is 75 µg/l and 55µg/l for females (Shortt et al., 1997). It is apparent that many European diets, including the UK diet, currently have an inadequate selenium content.

Country	Intake (µg per day)	Information source
UK	29–39	UK Ministry of Agriculture, Fisheries, Food, 1997
Belgium	28–61	Robberecht and Deelstra, 1994
France	29–43	Lamand and colleagues, 1994
Germany (Bavaria)	35	Kumpulainen and Salonen, 1996
Netherlands	67	Kumpulainen, 1993
Denmark	38–47	Danish Government Food Agency, 1995
Sweden	38	Kumpulainen, 1993
Switzerland	70	Kumpulainen, 1993
Poland	11–24 (estimate)	Kvícala and colleagues, 1995, 1997
Slovakia	38	Kadrabova, 1998

Table 8.1: Selenium intake in the European countries (Rayman, 2000).

This observations support the view that the lower selenium status found in diabetic patients is not the result of an acute phase response or diminished hepatic synthesis of plasma selenium-binding proteins due to disease. These data would suggest that

diabetic patients may benefit from selenium supplementation to optimize their antioxidant stores and thus diminish the harmful effects of oxidative stress.

Finally, we found that selenium concentrations in the serum of our patients (type II diabetes mellitus) and healthy volunteers groups were well below the recommended level that is required to achieve optimal expression of selenoenzymes in tissues (chapter seven). Importantly we found that selenium status and the expression of red cell GPX1 was inversely proportional to the BMI of the study subjects. This implies that, in this UK population, obesity is linked to a diminution in selenium status and selenoprotein expression; this in turn may predispose such obese individuals to tissue damage from increased oxidative stress. Because obesity also leads to insulin resistance, which is a known risk factor for the development of NAFLD, one could argue that regimes designed to help patients lose weight and increase their selenium intake would be beneficial as regards preventing NAFLD.

Conclusion

In conclusion, I have shown that it is possible to use C3A and LX2 cells as model systems to investigate a potential role for selenium in anti-oxidant protection and in modulating the fibrotic response to oxidant injury. Both these cell types can be grown under selenium-deficient conditions and the effect of selenium supplementation on selenoenzyme expression demonstrated. These same cell types can also be fat-loaded using a number of fatty acid substrates and the ability of selenium to protect against oxidant damage under different conditions of fat-loading can then be studied. In both cell types, selenium protects against oxidant damage caused by fat loading or t-BuOOH treatment. The same model systems were used to study α-1 pro-collagen mRNA expression. A novel finding was that C3A cells showed α-1 pro-collagen expression to be greatly stimulated by TGF-β1 treatment (especially in oleate-loaded cells) with an ability of selenium to reduce α-1 pro-collagen expression. LX2 cells appeared to be already extensively activated, with high basal levels of α-1 pro-collagen expression. The studies on diabetic subjects confirmed other work that the UK population is relatively selenium-deficient and also showed a significant negative association of selenium levels with BMI which could have important clinical implications. The work in this thesis raises important

questions as to the potential protective effect of selenium on hepatic injury and fibrosis in obese/diabetic subjects with fatty liver which warrant further clinical studies.

8.2 Future studies

Further studies are suggested to pursue different aspects of this thesis, and are as follows:

1. Studies on the relationship between α-1 pro-collagen expression in C3A cells and their transformation to myofibroblast-like cells should be performed. This should be addressed at the protein level to investigate the signalling pathways thought to be involved in the cell transformation. The influence of selenium on these signalling pathways should also be studied.

2. Investigate the means by which it is possible to convert LX-2 cells to a quiescent state. This would allow the model to be used to look at the mechanism of activation of stellate cells and determine if selenium status could modify this, as is the case for C3A cells.

3. Investigate the mechanism(s) by which t-BuOOH treatment decreases α-1 pro-collagen expression in both C3A and LX-2 cells in relationship to oxidative stress and subsequent selenoenzyme expression.

4. Establish a dietary selenium-supplementation trial in an at risk cohort of patients with obesity or type 2 diabetes to assess the effect of selenium supplementation on the development of NAFLD and liver fibrosis.

9 CHAPTER NINE

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Appendices

A3 (Chapter three appendix)

A3.1 Methods

General protocol
For experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in section 2.2.1. The cells were passaged and re-plated into two 6-well (35 mm) or 12 well plates for each cell type. After 24 hr incubation (i.e. at confluence) treatments was started as described below.

Protocol A3.1.1: The medium was changed to a variety of different supplements in DMEM for 48 hours, including: 10 % FBS, BSA (ranging from 0-0.75% addition) or DMEM with insulin [10 µg/ml] plus transferrin [5 µg/ml]. After incubation for 48 hours, 0.5 ml of conditioned medium from the wells was collected into a pre-labelled 2 ml Eppendorff tube and centrifuged at 500 x g for 5 min to remove cells and cell debris. The LDH content of the medium was then measured as described in section 2.2.4 as a measure of cell viability.

Protocol A3.1.2: The medium was changed for a further 48 hours as follows: 10 % FBS-supplemented medium (as a positive control of maintenance of cell viability), medium with different combinations of added insulin/transferrin [0/0, 2.5/1.25, 5/2.5, 10/5, 20/10µg/ml]. To assess cell viability, the medium and the cells were collected as described in section 2.2.2. LDH assays were carried out on both cells and media according to the method described in section 2.2.4 to calculate percentage LDH retention as a measure of cell viability.

Protocol A3.1.3: The medium was changed to the following conditions for 48 hours: 10 % FBS-supplemented DMEM medium (as a positive control to maintain cell viability), DMEM medium with insulin added at concentrations over the range0, 2.5, 5, 10, 20 µg/ml and DMEM medium plus transferrin added over the concentration

range0, 1.25, 2.5, 5, µg/ml. Both cells and media were collected and cell viability assessed by LDH retention.

Protocol A3.1.4: The medium was changed to the following supplementations: 10 % FBS-supplemented DMEM medium (positive control), DMEM medium with added transferrin at concentrations ranging from 0, 10, 50, 100, 500 µg/ml for 48 hours.

Protocol A3.1.5: The medium was changed to the following supplementation: DMEM medium with added 'in-house' IT plus selenium (10 µg/ml (I) /5 µg/ml (T), 58 nM (selenium)) and commercial ITS (10 µg/ml (I) /5 µg/ml (T), 58 nM (selenium)) for 72 hours. After incubation, cells were collected and thioredoxin reductase1 protein levels measured as described in sections 2.2.2 and 2.2.8 respectively. Protein concentrations were measured using the Bradford assay (section 2.2.5).

A3.2 Results

A3.2.1

A3.2.2 Effect of bovine serum albumin (BSA) supplementation on C3A cell viability:

FBS-free medium is commonly supplemented with BSA to help maintain a protein balance for tissue culture. Another supplement frequently used to maintain cell viability is ITS (Insulin, Transferrin and Selenium). To assess whether either of these supplements aided the cell viability in the absence of FBS, protocol A3.1 was followed.

The initial experiment was directed at the use of BSA supplementation because of a concern that the inclusion of insulin might confound later aspects of the study because of its association with fatty liver disease. Various concentrations of BSA supplementation were tested while IT was tested at its commercially recommended concentrations of insulin [10 µg/ml] plus transferrin [5 µg/ml]. As shown in figure A3.1, inclusion of BSA in the absence of IT had a minimal effect on maintaining cell viability. However, when IT-supplementation with albumin was used LDH release was minimised.

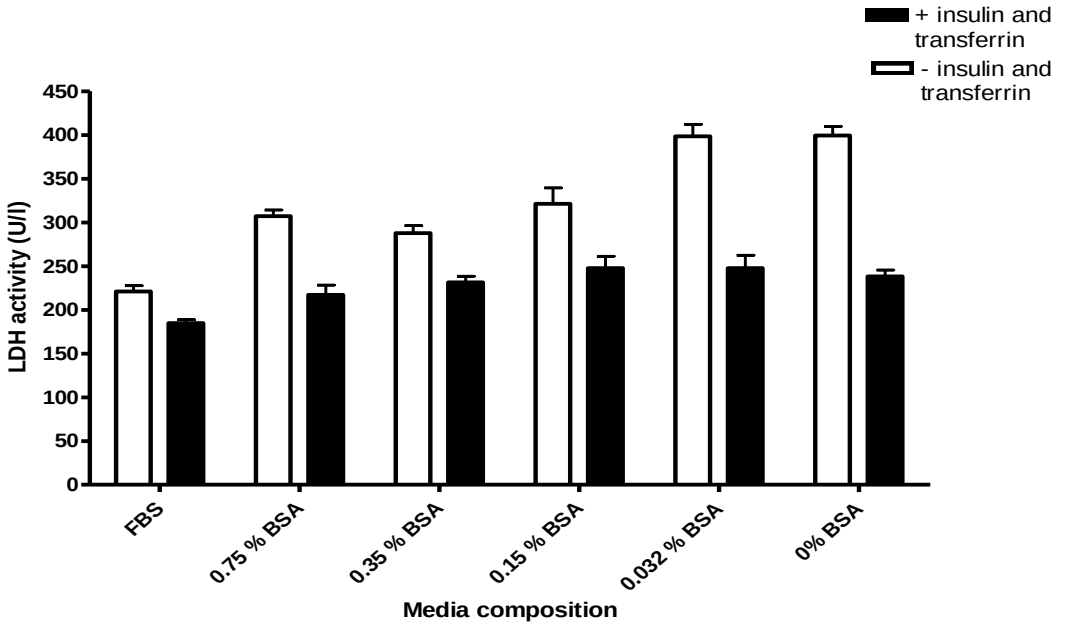


Figure A3.1: The effect of bovine serum albumin (BSA) plus or minus insulin and transferrin on the LDH released from C3A cells.

C3A cells were plated in FBS-supplemented medium and after 24 hours, medium was changed to FBS-supplemented medium, medium (minus FBS) with added BSA in various concentrations ranging from (0-0.75%) plus or minus insulin (10 µg/ml) and transferrin (5 µg/ml), respectively for 48 hours. Harvested media were assessed for LDH. Data shown are from a single experiment done with quadruplet wells.

A3.2.3 The effect of insulin and transferrin supplementation on the cell viability and thioredoxin reductase expression in C3A and LX-2 cells:

Commercial ITS supplement is frequently used to maintain cell viability when serum-deficient medium is required. To assess whether insulin/transferrin alone (no BSA) is sufficient to maintain cell viability, preliminary experiments on C3A and LX-2 cells were carried out according to protocol A3.1.2.

As shown in figures A3.2B and A3.3B, IT supplementation alone (i.e. without BSA) is capable of maintaining the viability of both cell types. However, the effect does not appear to alter with the different ratios of I/T used in this experiment. It was therefore necessary to determine which of the IT components (and at what concentration), was more effective in maintaining cell viability as described in protocol A3.1.3.

As shown in figures A3.2C and A3.3C, transferrin had little effect on cell viability, whereas, insulin was effective at maintaining cell viability, even at the lowest concentration used [2.5 ug/l] (see figure A3.2A).

Further experiments explored whether transferrin in higher doses was more effective in maintaining cell viability, as it is commonly believed that a high medium protein concentration is necessary to maintain cell membrane viability. Similar experiments to those described above were conducted as described in protocol A3.1.4,

Figures A3.2D and A3.3D demonstrate that transferrin added alone at higher doses did not facilitate maintenance of cell viability.

At this stage of the investigation, it was important, to verify that IT supplementation did not affect the thioredoxin reductase 1 expression. As shown in figures A3.4 and A3.5 there was no effect of IT supplementation on the level of thioredoxin reductase compared to positive and negative controls of FBS-supplemented and basic medium respectively.

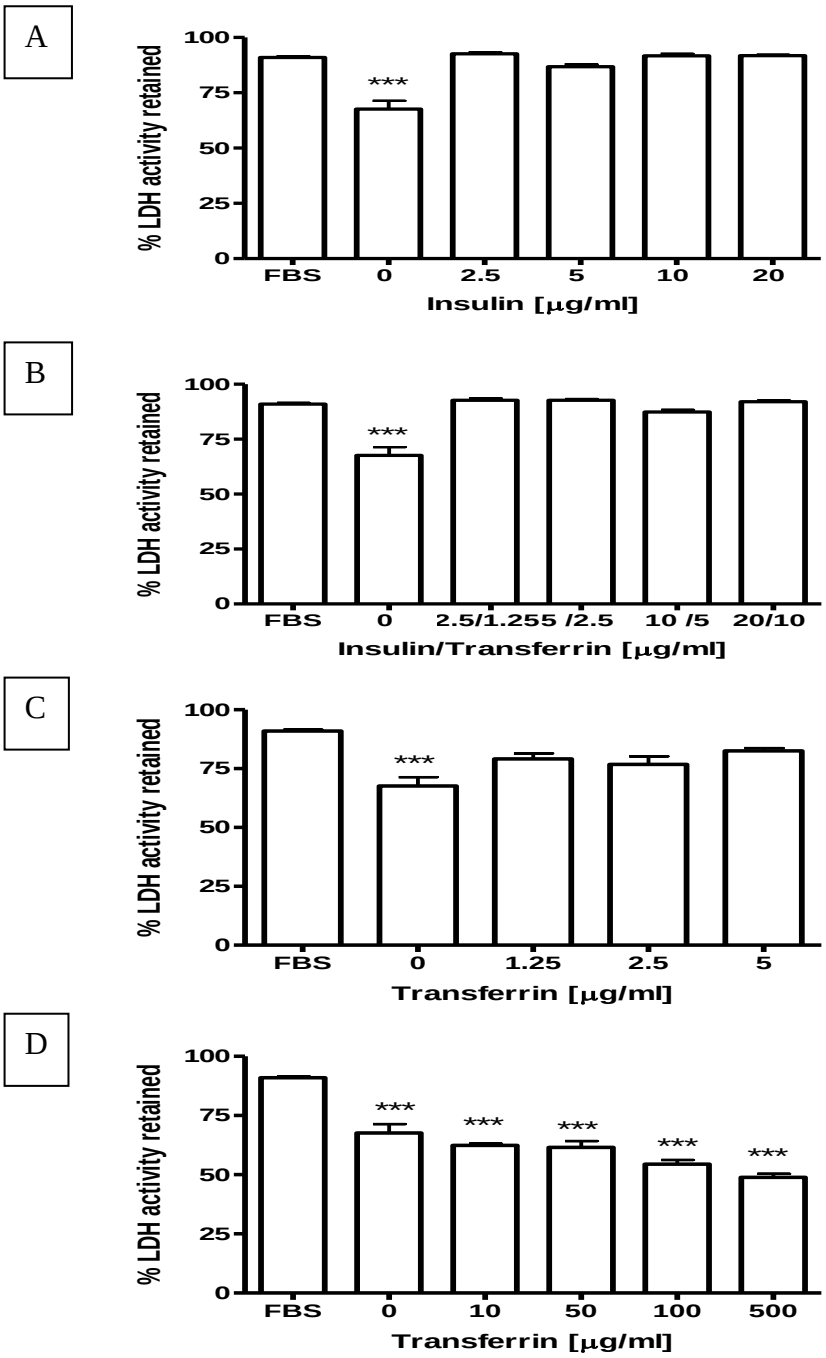


Figure A3.2: The effect of (A) insulin, (B) insulin plus transferrin, (C) transferrin, low concentration range and (D) transferrin, high concentration range on the percentage of lactate dehydrogenase activity (%LDH) retained in C3A cells. Cells were cultured in FBS-supplemented medium and, after 24 hours, medium was changed to FBS-supplemented medium (positive control), medium with added insulin (A); medium with a combination of insulin and transferrin (B); medium with transferrin with a low (C) or high (D) concentration range. After 48 hours, cells and media were collected and assessed for LDH described in section 2.2.4. Data shown is for a single experiment with each data point determined with quadruplicate wells (mean ± SEM). *** $p<0.001$ compared to FBS.

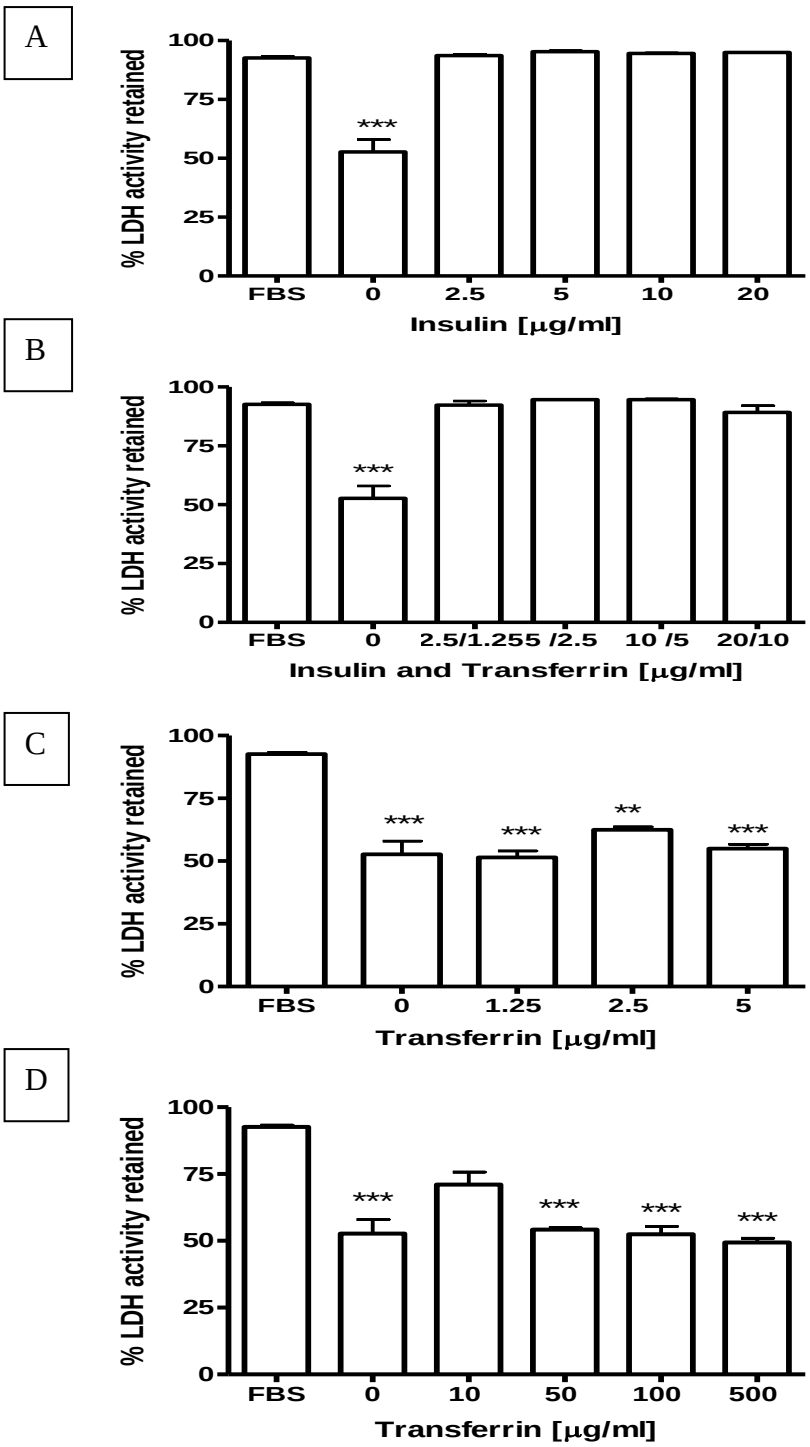


Figure A3.3: The effect of (A) insulin, (B) insulin plus transferrin, (C) transferrin (low concentration range) and (D) transferrin (high concentration range) on the percentage lactate dehydrogenase activity (%LDH) retained in LX-2 cells. The experimental conditions were as described for the C3A cells in figure A3.2 (mean ± SEM). ** $p<0.01$, *** $p<0.001$ compared to FBS.

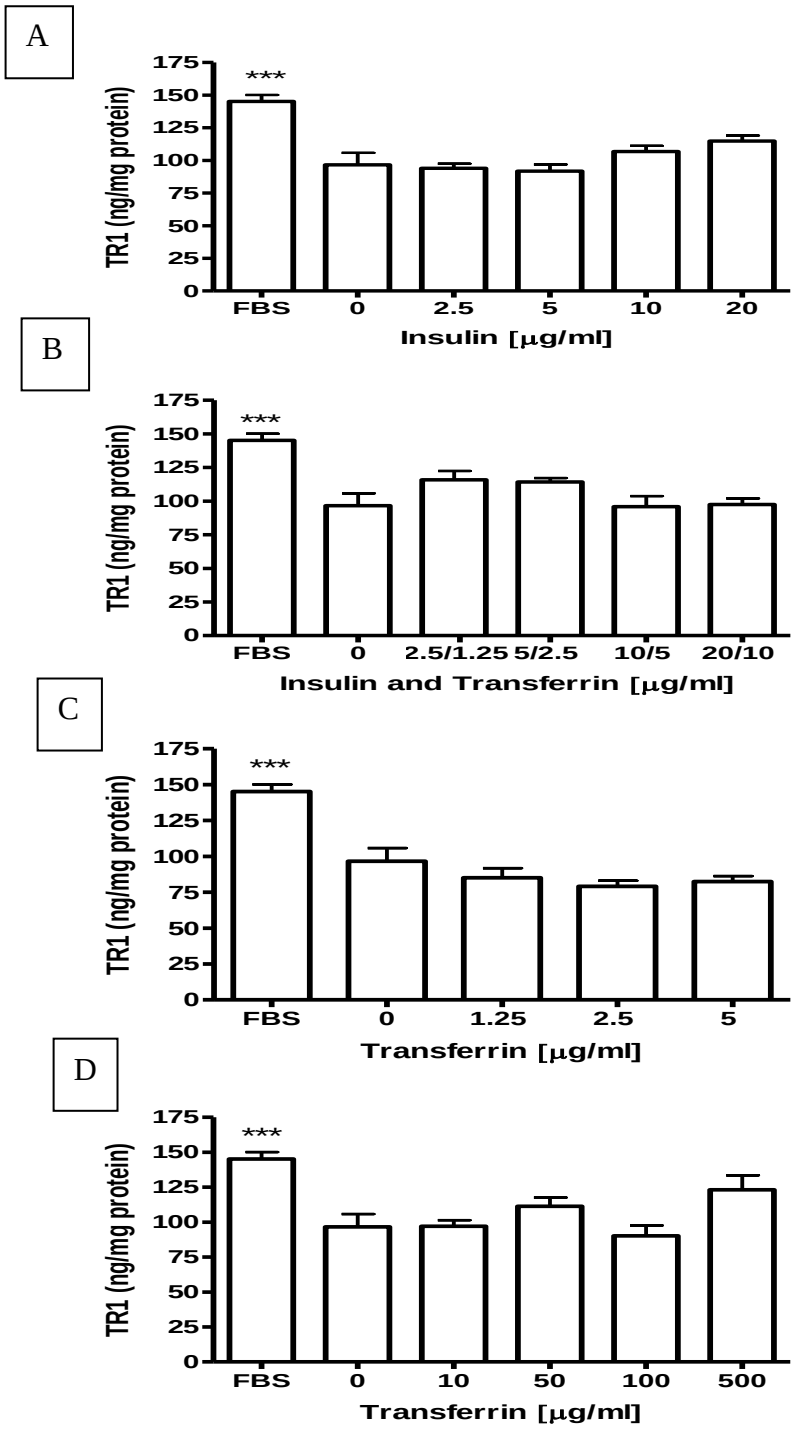


Figure A3.4: The effect of (A) insulin, (B) insulin plus transferrin, (C) transferrin, low concentration range and (D) transferrin, high concentration range on thioredoxin reductase 1 (TR1) in C3A cells. Cells were cultured in FBS-supplemented medium and after 24 hours, medium was changed to FBS-supplemented medium; (A) medium with added insulin; (B) combinations of insulin and transferrin; (C) transferrin (low concentration range); (D) transferrin (high concentration range). Cells were incubated for 48 hours. Data shown are from a single experiment with each data point analysed from determinations in quadruplicate wells (mean ± SEM). *** $p<0.001$ compared to 0 concentration of (A) insulin/transferrin, (B) insulin, (C), (D) transferrin.

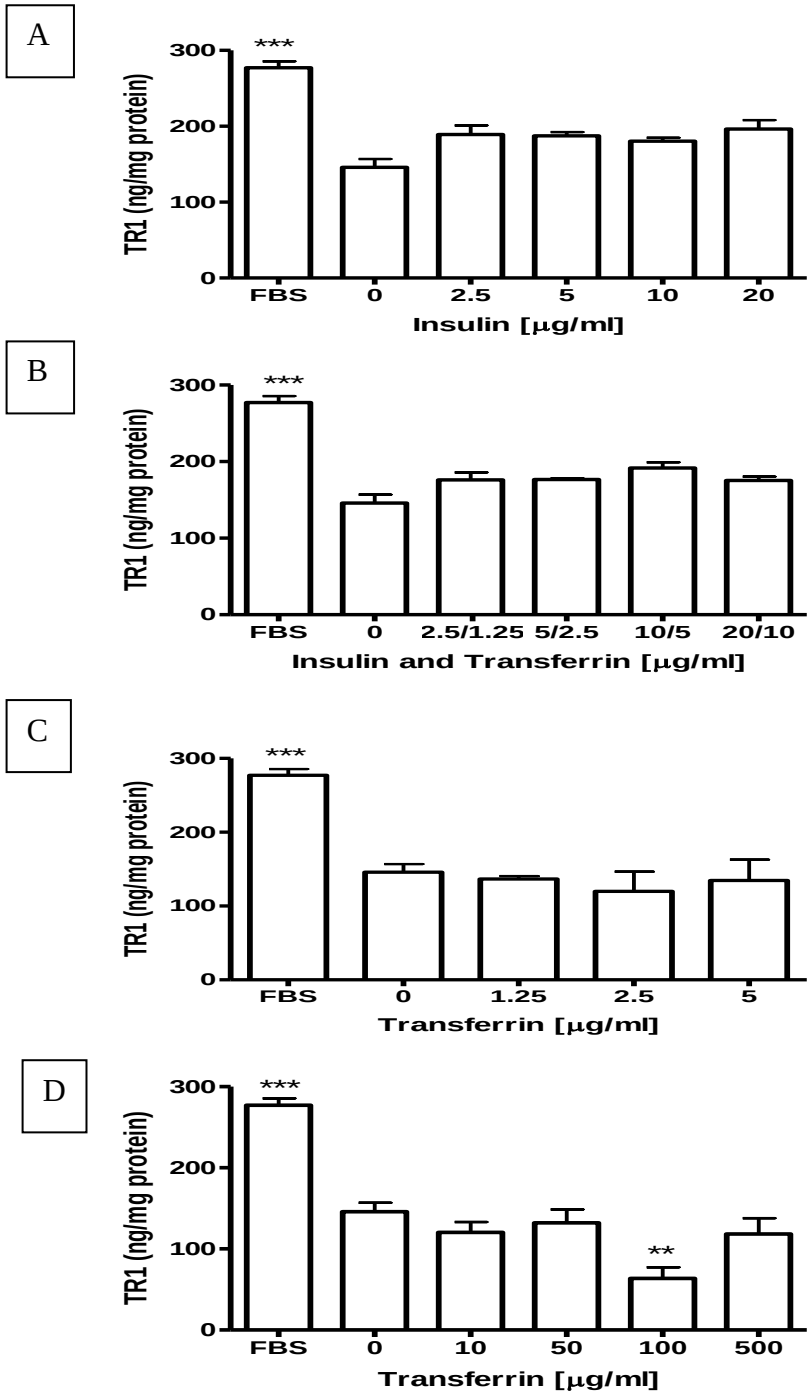


Figure A3.5: The effect of (A) insulin, (B) insulin plus transferrin, (C) transferrin (low concentration range) and (D) transferrin (high concentration range) on thioredoxin reductase 1 (TR1) levels in LX-2 cells. The experimental conditions were as described in Figure A3.4. Data shown are from a single experiment with each data point arising from quadruplicate determinations (mean ± SEM). *** $p<0.001$, ** $p<0.01$ compared to 0 concentration of (A) insulin/transferrin, (B) insulin, (C), (D) transferrin.

A3.2.4 Comparison of the 'in-house' IT plus selenium with commercially sourced ITS:

This experiment was conducted to compare the effect of 'in-house' prepared IT supplementation (with and without selenium) with commercially obtained ITS on TR1 expression according to protocol A3.1.5.

As shown in figure A3.6, for C3A cells no significant difference in thioredoxin reductase 1 response between 'in-house' and commercial ITS preparations was seen.

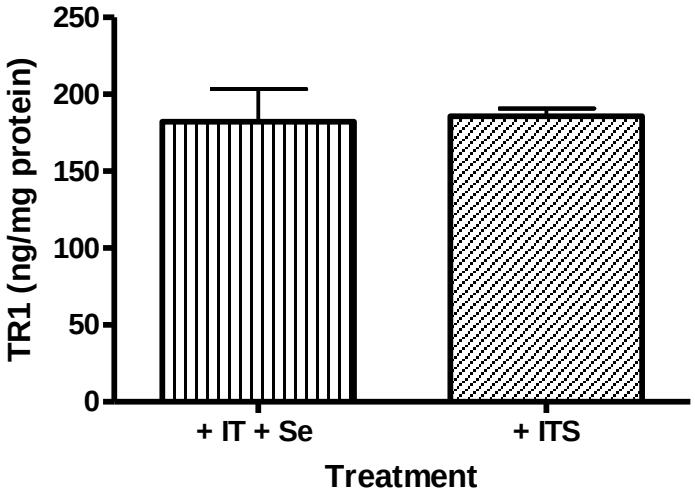


Figure A3.6: Comparison between the commercially available ITS supplement and IT plus selenium prepared 'in house'.
C3A cells were plated in DMEM medium containing 10 % FBS. After 24 hours, different supplements (as shown on the graph) were added for 72 hours. Cells were then harvested. TR1 level was measured as described. IT+ Se refers to the 'in-house' selenium supplemented IT medium, while ITS refers to the commercially available supplement. This experiment was done once (each data point determined using triplicate wells (mean ± SEM)).

A4 (Chapter four appendix)

A4.1 Methods

General protocol: For experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in section 2.2.1. After 24 hr incubation (i.e. at confluence) treatments was started as described below.

Protocol A4.1.1: The cells were treated with oleate or palmitate at various concentrations [2 mM], [1 mM] or [0.5 mM] and then cultured for 24 hours. The percentage LDH retained was measured as described in section 2.2.4.

Protocol A4.1.2: The medium was changed to IT-supplemented medium for a further 24 hours. Cells were then washed twice with HBSS before treatment with oleate [0.25 mM] or palmitate [0.125 mM] contained in IT-supplemented medium for different incubation periods of 24, 48 or 72 hours. Cells were then collected and triglyceride content assessed as described in section 2.2.6.

Protocol A4.1.3: The medium was changed to IT-supplemented medium after 24 hours of plating for a further 24 hours. Cells were washed twice with HBSS and then treated for 24 hours with combination of oleate/palmitate (2/1) ([0.166 mM]/[0.083 mM]) and ([0.33 mM]/[0.166 mM]) at a total fatty acid concentration of [0.25 mM] and [0.5 mM] respectively contained in IT-supplemented medium. Cells were collected and their triglyceride content assessed as described in section 2.2.6.

Protocol A4.1.4: The medium was changed to an IT-supplemented medium for further 24 hours. Cells were then washed twice with HBSS before treatment with lactate [20 mM], pyruvate [1 mM], octanoate [4 mM] or NH_4Cl [4 mM] or LPON supplemented-IT medium for 48 hours. Cells were then collected and triglyceride content assessed as described in section 2.2.6. TR1 levels were assessed as described in section 2.2.8. GPX1 activity was assayed as described in section 2.2.3.2.

Protocol A4.1.5: The medium was changed to IT-supplemented medium (for 24 hours) 24 hours after initial plating. Cells were then washed twice with HBSS followed by treatment with either oleate [0.25 mM] or palmitate [0.125 mM] for 24 hours, or LPON for 48 hours. All treatments were in IT-supplemented medium. Cells were then collected and cholesterol content assessed as described in section 2.2.7.

Protocol A4.1.6: The cells received IT-medium for 24 hours. After that, two groups of LX-2 cells were treated with oleate [0.25 mM] in IT-medium for 24 hours. Both groups of cells were then washed twice with HBSS and then one group was treated with TGF-β1 [2.5 ng/ml] for a further 24 hours in IT. The other group of cells (oleate-loaded cells only - as a positive control) received IT-supplemented medium for further 24 hours. Control cells also received IT-supplemented medium that was changed every 24 hours.

Protocol A4.1.7: The corresponding experiment was conducted in the reverse order when cells were first treated by TGF-β1 [2.5 ng/ml] for 24 hours. 24 hours later, cells were treated with oleate [0.25 mM] for a further 24 hours. Control cells and oleate-loaded cells were included, where control cells received IT-supplemented medium that was changed every 24 hours. Oleate-loaded cells received IT-supplemented medium for the first 24 hours followed by treatment with oleate [0.25 mM] for 24 hours.

Protocol A4.1.8: LX-2 cells were treated with both oleate [0.25 mM] and TGF-β1 [2.5 ng/ml] simultaneously for 24 hours. Control cells and oleate-loaded cells were included, control cells receiving IT-supplemented medium that was changed every 24 hours. Oleate-loaded cells were treated with oleate [0.25 mM] for 24 hours. Cells were washed twice with HBSS and then triglyceride content was assessed as described in section 2.2.6.

A4.1.1 The effect of oleate and palmitate treatment on cell viability

To determine the optimal dose of oleate or palmitate that could induce significant triglyceride accumulation in C3A and LX-2 cells without affecting their viability, protocol A4.1.1 was conducted.

As shown in figure A4.1, both C3A and LX-2 cells were unable to survive at concentrations of 2 mM oleate. Unlike the C3A cells, LX-2 cells were able to accommodate 1 mM oleate. Although, 0.5 mM oleate appeared not to affect cell viability, it was decided that further experiments would be carried out using lower concentration of the fatty acid.

As shown in figure A4.2, palmitate-loaded cells showed compromised viability with 0.5 mM fatty acid addition. Thus, on a molar basis, palmitate is more toxic than oleate and may exert more acute injury to cells. This is in agreement with the findings of Gomez-Lechon et al (Gomez-Lechon et al., 2007).

LX-2 cells appear to be more resistant to oleate and palmitate than C3A cells. Although, 1mM oleate or palmitate seems to be a toxic dose for C3A cells, this concentration had little effect on the viability of LX-2 cells. Both cell types were not able to survive at 2 mM oleate or palmitate.

In subsequent experiments, 0.5mM and lower concentrations of oleate and 0.25 mM and lower doses of palmitate were used.

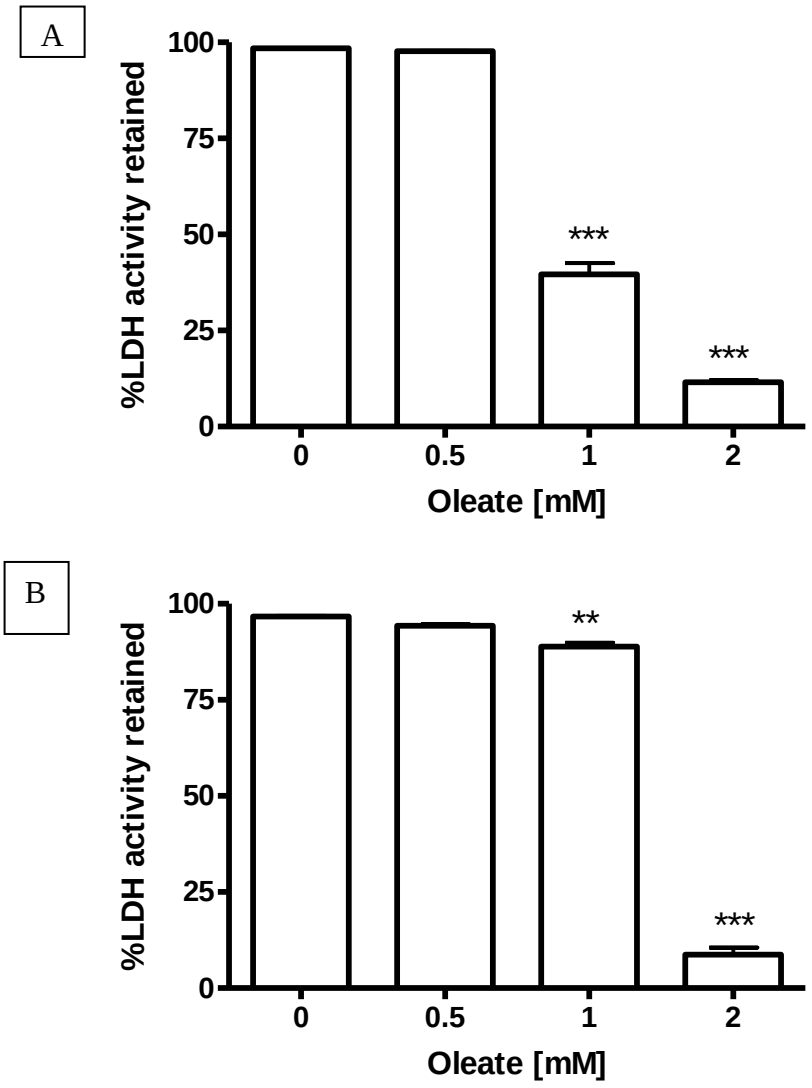


Figure A4.1: The effect of oleate on the viability of (A) C3A and (B) LX-2.
C3A and LX-2 cells were incubated with oleate at various concentrations for 24 hours. Cells were harvested and media collected to assess the %LDH activity retained by cells. Data shown represent a single experiment carried out using triplicate wells (mean ± SEM). ** $p<0.01$, *** $p<0.001$ compared to 0 concentration of oleate.

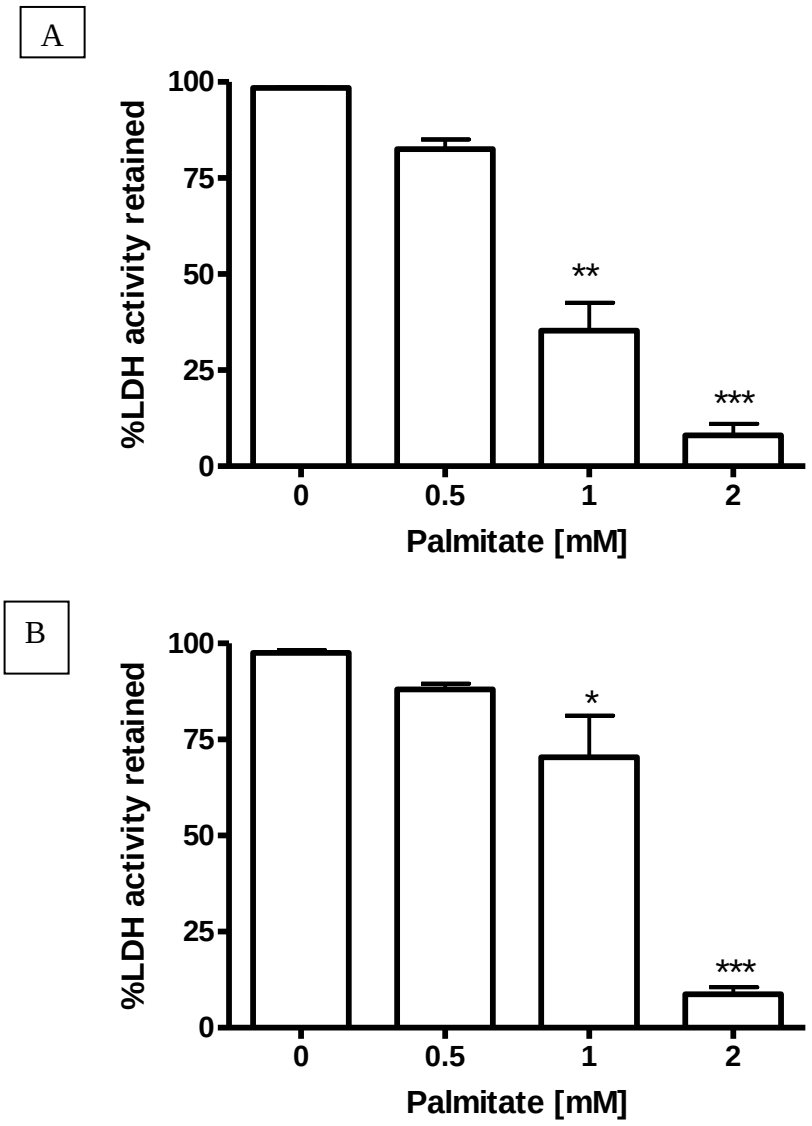


Figure A4.2: The effect of palmitate on the viability of (A) C3A and (B) LX-2. C3A and LX-2 cells were incubated with palmitate at various concentrations for 24 hours. Cells were harvested and media were collected to assess the %LDH activity retained in the cells. Data shown represent a single experiment conducted using triplicate wells (mean ± SEM). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to 0 concentration of palmitate.

A4.1.2 The effect of varying incubation time on oleate or palmitate fat-loading

To further investigate and determine the optimal incubation period to observe triglyceride accumulation, protocol A4.1.2 was followed.

As shown in figure A4.3, the maximal triglyceride accumulation in C3A and LX-2 cells was attained after 24 hours incubation.

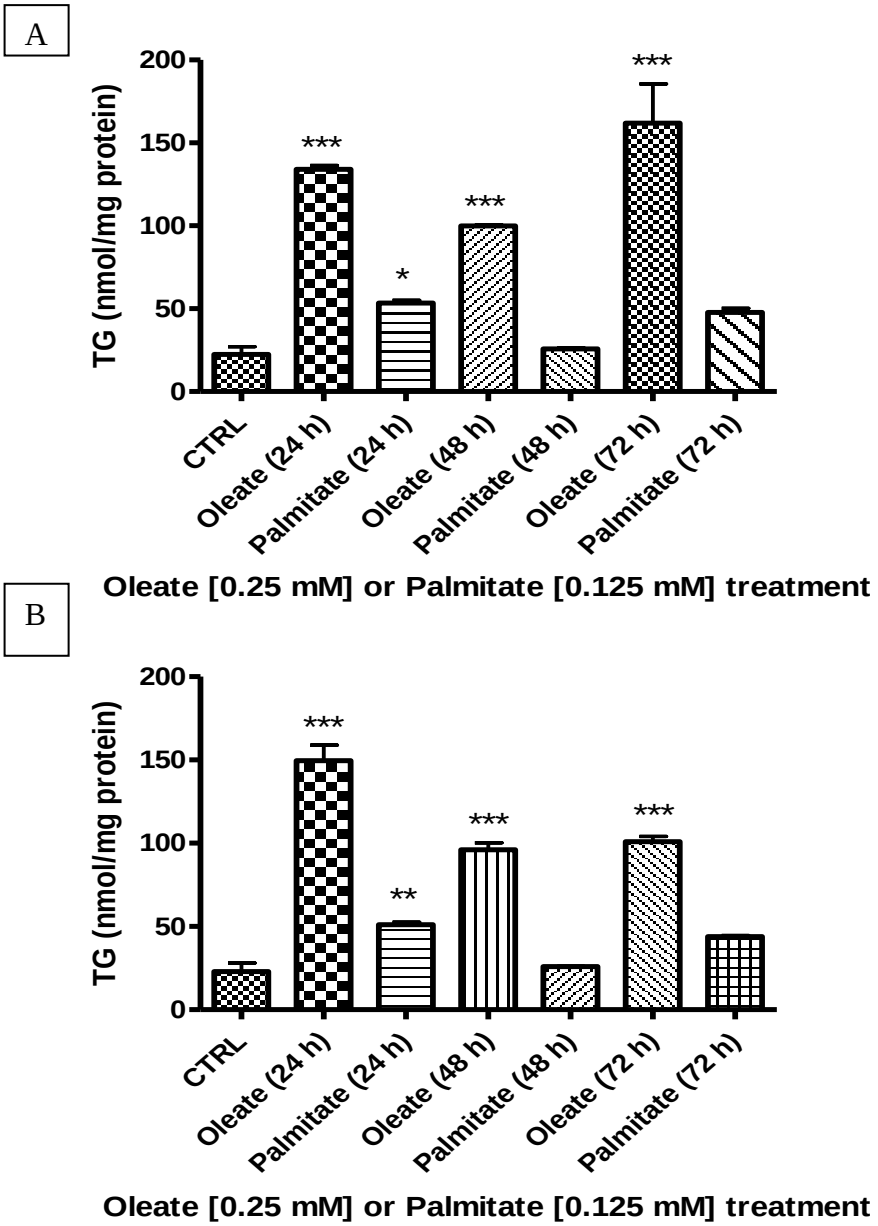


Figure A4.3: Effect of incubation time on (A) C3A and (B) LX-2 after exposure to oleate or palmitate. C3A and LX-2 cells were incubated with either oleate [0.25 mM] or palmitate [0.125 mM] for 24, 48 or 72 hours. Cells were then harvested and triglyceride content was assessed as described in section 2.2.6. Data represent the mean ± standard error (± SEM) n=3 for both A and B, each experiment done in triplicate. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to CTRL (no fatty acid addition).

A4.1.3 The effect of combined oleate and palmitate addition on fat loading

The effect on triglyceride accumulation of the addition of both oleate and palmitate was also studied. The experiment was conducted as described in protocol A4.1.3.

As shown in figure A4.4, both C3A and LX-2 responded to the addition of the fatty acid combination of oleate/palmitate (2/1) by accumulating triglyceride in a degree related to the total concentration of the supplemented fatty acids. Oleate was chosen to comprise two thirds of the final concentration of the combination because oleate was found to have less adverse effect on cell viability as compared to palmitate. Moreover, palmitate was claimed to induce acute injury whereas oleate treated cells would lead to more chronic injury (Gomez-Lechon et al., 2007).

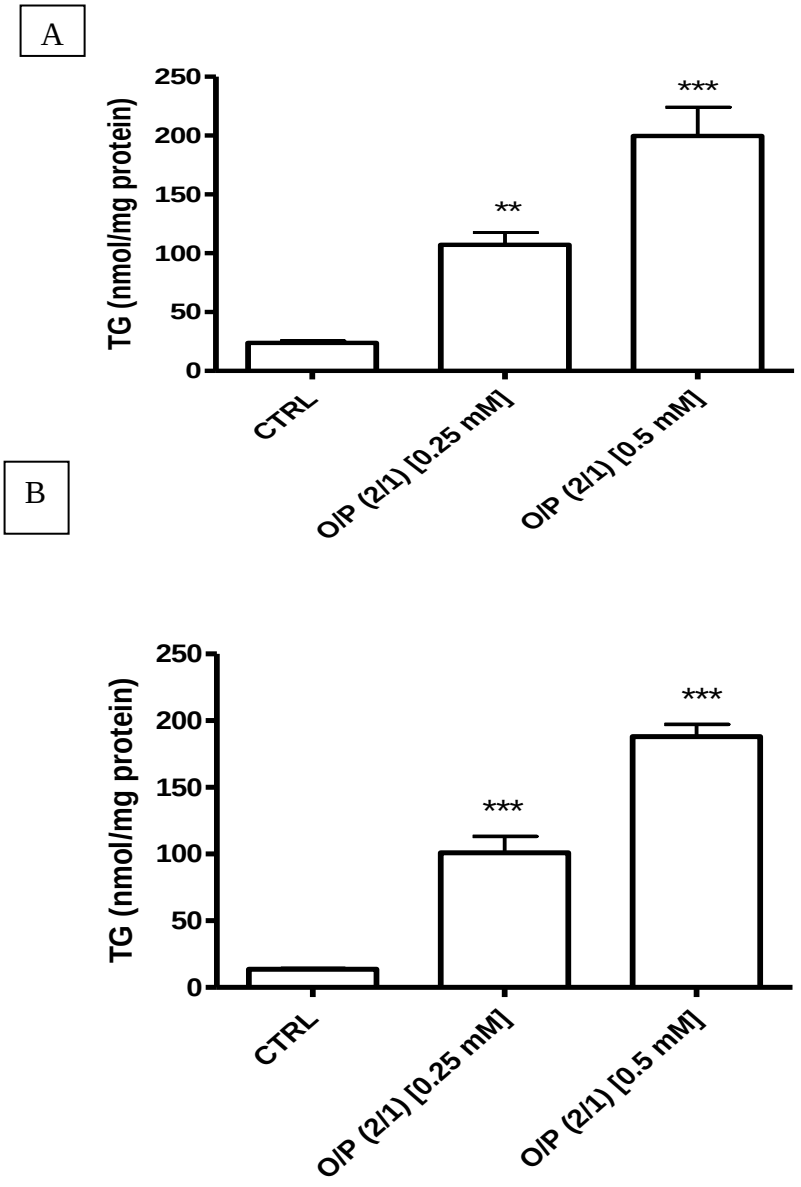


Figure A4.4: Triglyceride content of (A) C3A and (B) LX-2
C3A and LX-2 cells were incubated with combinations of oleate/palmitate (2/1) at a total concentrations of [0.25 mM]/ [0.5 mM], respectively, contained in IT-supplemented medium for 24 hours. Cells were then collected and triglyceride content was assessed according to the method described in section 2.2.6. Data represent the mean (± SEM) n=3 for both A and B, with each experiment done in triplicate. ** $p<0.01$, *** $p<0.001$ compared to CTRL (no fatty acid addition).

A4.1.4 Effect of LPON treatment of C3A and LX-2 cells in FBS- or IT-supplemented medium

After a suitable selenium-deficient medium was identified (i.e. IT-supplemented medium), it was important to examine the effect of marked selenium deprivation on triglyceride accumulation and selenoenzyme expression. For this purpose, protocol A4.1.4 was conducted.

As illustrated in figures A4.5, A4.6 and A4.7, the triglyceride accumulation could be achieved in selenium-deficient medium (IT-medium). The triglyceride level was similar to the level obtained when these experiments were done in selenium replete (10% FBS-supplemented medium)(see figures 4.4, 4.5 and 4.6). This was true for both LX2 and C3A cells. This suggests that IT-supplemented medium could be used instead of FBS-supplemented medium when a fat-loading cell in a completely selenium-deficient environment is required, but without any compromise of cell viability.

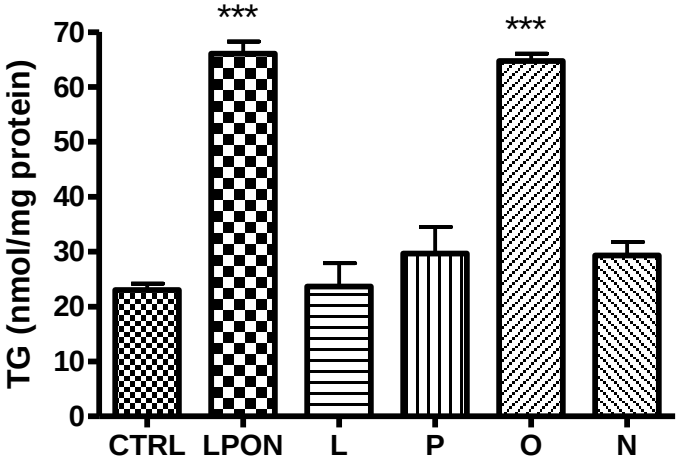
When comparing experiments done in selenium-deficient condition with the selenium-replete setting, the triglyceride levels of the control cells were very similar (25 ± 5 and 30 ± 5 nmole/mg protein, respectively). The triglyceride levels of cells treated with LPON or octanoate showed lower levels of triglyceride in a selenium deficient setting (reducing from 90 ± 6 to 65 ± 7 nmole/mg protein for C3A cells and from125 ± 5 to 55 ± 8 nmole/mg protein for LX-2 cells). Other components of LPON were not significantly different to control cells for both C3A and LX-2 cells.

The TR1 levels (as expected) were lower in control cells grown in IT-medium compared to cells grown in 10 % FBS supplemented medium (reducing from 95 ± 6 to 60 ± 10 ng/mg protein (C3A cells) and from 180 ± 12 to 85 ± 8 ng/mg protein (LX-2 cells). The TR1 response to LPON or octanoate was also different in a selenium-deficient setting compared to a selenium-replete condition, reducing from about 150 % to about 160 % of control cells for C3A cells and from about 200 % to

about 134% for LX-2 cells. Lactate/pyruvate or ammonium components of LPON were not significantly different to control cells in both C3A and LX-2 cells.

The GPX1 level (also as expected) were lower in control cells grown in selenium deficient conditions compared to cells grown under selenium replete conditions (reducing from 25 ± 4 to 2.5 ± 0.2 mU/mg protein for C3A cells and from 30 ± 5 to 2.5 ± 0.3 mU/mg protein for LX-2 cells). Although, lower levels of GPX1 were observed in all treatments compared to the selenium replete situation, the GPX1 response to LPON or octanoate was higher compared to selenium-repletion condition (increasing by about 160% in selenium replete conditions as compared to 480% in selenium deficiency for C3A cells and from about 160% in selenium replete conditions to about 370% in selenium deplete conditions for LX-2 cells this is confused). Lactate/pyruvate or ammonium components of LPON were not significantly different to control cells in both C3A and LX-2 cells.

A



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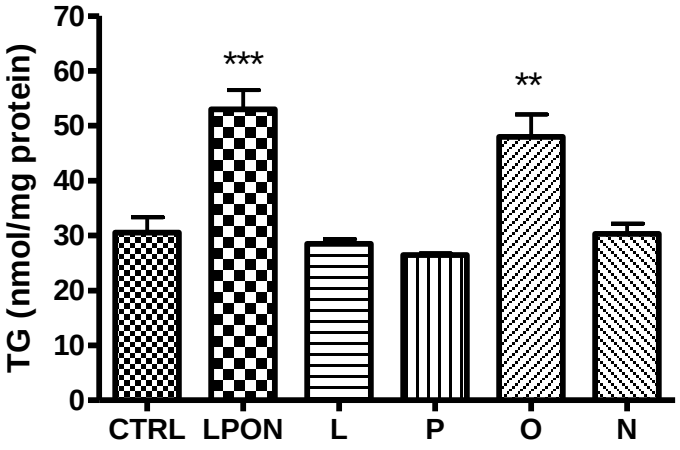

Figure A4.5: The effect of LPON and its separate components on triglyceride content of (A) C3A and (B) LX-2 in IT-supplemented medium. C3A and LX-2 cells were pre-incubated in IT-supplemented medium for 24 hours. Cells were then incubated for 48 hours with LPON and its separate components (contained in IT-supplemented medium). Cells were then collected and triglyceride content assessed as described in section 2.2.6. Data represent one experiment done in triplicate. L=Lactate, P=Pyruvate, O=Octanoate, N= ammonium chloride (NH4CL). Data represent the mean ± standard error (± SEM), ** $p<0.01$, *** $p<0.001$ compared to CTRL (no addition of any components of LPON).

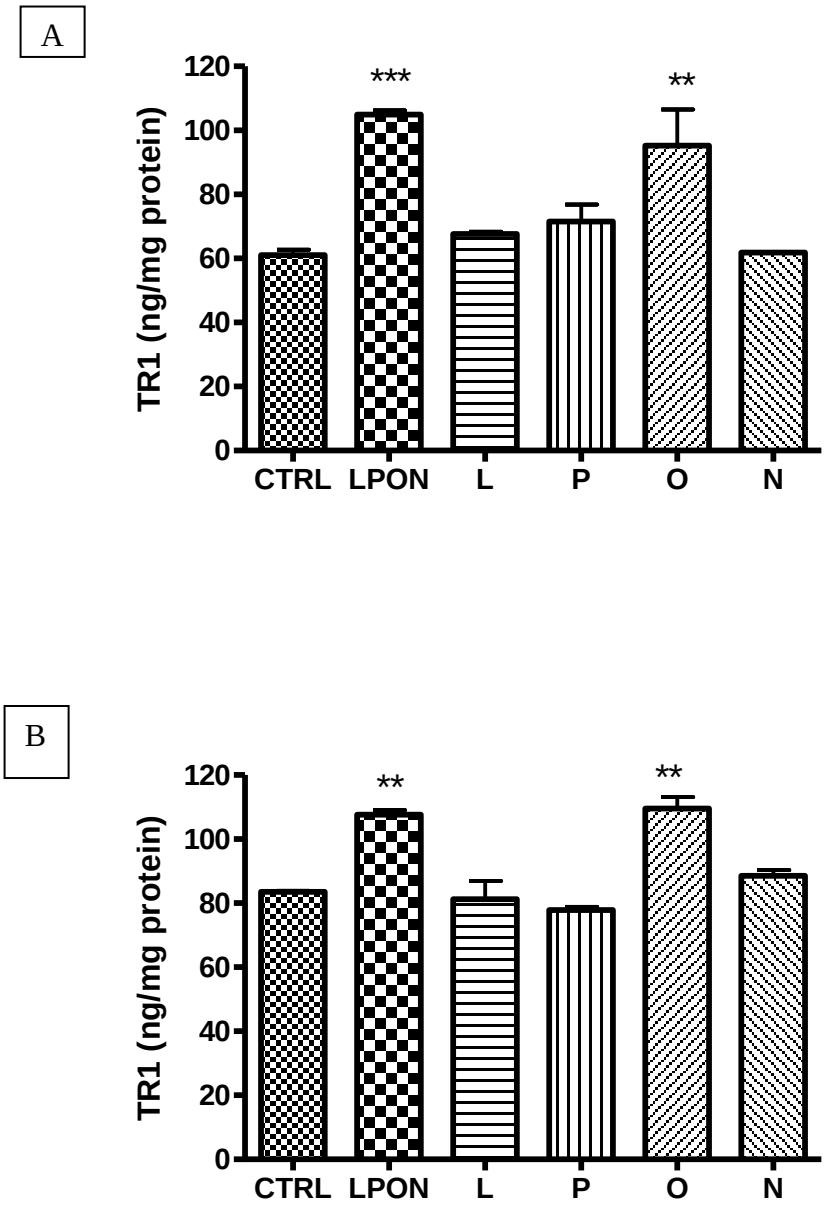

Figure A4.6: The effect of LPON and its various components on TR1 levels in IT-supplemented media setting of (A) C3A and (B) LX-2. C3A and LX-2 cells were pre-incubated in IT-supplemented medium for 24 hours. Cells were then incubated for 48 hours with LPON or its separate components (contained in IT-supplemented medium). Cells were then collected and TR1 assessed as described in section 2.2.8. Data represent one experiment done in triplicate. L=Lactate, P=Pyruvate, O=Octanoate, N= ammonium chloride (NH4CL). Data represent the mean ± standard error (± SEM) for 3 wells, ** $p<0.01$, *** $p<0.001$ compared CTRL (no addition of any components of LPON).

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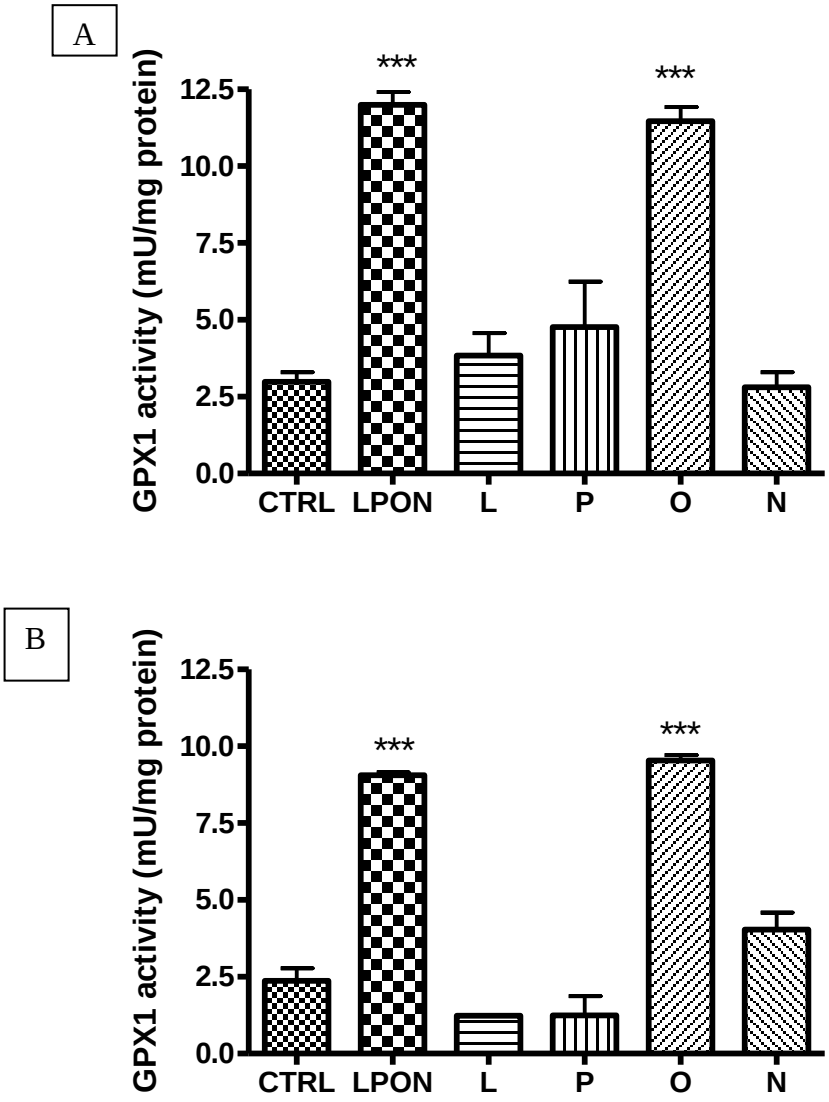

Figure A4.7: The effect of LPON and its various components on GPX1 activity of (A) C3A and (B) LX-2 in IT-supplemented medium. C3A and LX-2 cells were pre-incubated in IT-supplemented medium for 24 hours. Cells were then incubated for 48 hours with LPON or its separate components (contained in IT-supplemented medium). Cells were then collected and GPX1 assessed as described in section 2.2.3.2. Data represent one experiment done in triplicate. L=Lactate, P=Pyruvate, O=Octanoate, N= ammonium chloride (NH4CL). Data represent the mean ± standard error (± SEM), *** $p<0.001$ compared to CTRL (no addition of any components of LPON).

A4.1.5 Cholesterol levels of fat-loaded cells

To further explore the composition of lipid accumulated in C3A and LX-2 cells after treatment with LPON, oleate or palmitate, a pilot experiment was conducted according to protocol A4.1.5.

As shown in figure A4.8, the cholesterol content of cells did not change in contrast to their cellular triglyceride content.

This suggests that the lipids accumulating in LX-2 cells were mainly triglyceride upon treatment with fatty acids and LPON.

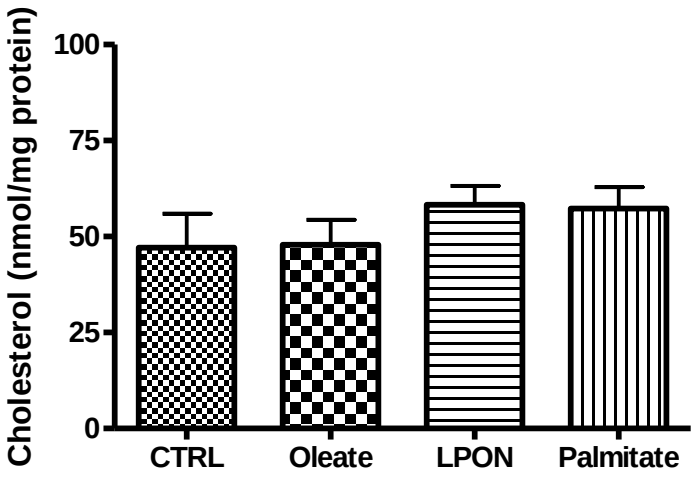

Figure A4.8: Cholesterol content of LX-2 cells treated with various fatty acids. LX-2 cells were incubated with oleate or palmitate for 24 hours and LPON for 48 hours. Cells were then collected and their cholesterol content assessed according to the method described in section 2.2.7. Data represent a single experiment. CTRL (no-addition).

A4.1.6 Loss of fat by TGF-β1-activated stellate cells

Hepatic stellate cells are normally quiescent and store vitamin A. However, in liver injury, they undergo increased cellular proliferation and phenotypically transform into a highly contractile cell type known as the myofibroblast. The activated phenotype is associated with loss of stored vitamin A (Friedman et al., 1993), increased synthesis of collagen and alpha-smooth muscle actin, increased contractility and increased deposition of extracellular matrix components (Friedman, 2000). Because LX-2 cells could be successfully fat-loaded, we proceeded to examine whether fat-loaded LX-2 cells might exhibit the loss of these fat droplets upon activation, as seen *in vivo*.

A4.1.6.1 The effect of TGF-β1 on the triglyceride content of normal and fat-loaded LX-2 cells:

Because the mechanism of stellate cell activation is poorly understood, I explored the activation process in relation to fat droplet loss, in three ways. Firstly, LX-2 cells were fat-loaded (perhaps achieving a quiescent status) and then cells were treated with the potent stellate cell activator cytokine, TGF-β1. Secondly, LX-2 cells were initially activated by TGF-β1, and then fat-loaded. Thirdly, LX-2 cells were simultaneously fat-loaded and treated with TGF-β1 for 24 hours according to protocols A4.1.6, A4.1.7 and A4.1.8 respectively.

Since oleate was less toxic and accumulated relatively more triglyceride than palmitate (see sections A4.1.2), oleate was chosen to fat-load hepatic stellate cells in these experiments.

As shown in figure A4.9, there was no change in the triglyceride content of LX-2 cells in response to TGF- β1 even though different strategies were used as mentioned above.

Further experiments were conducted in various different ways to try to demonstrate a loss of fat upon activation. Different fatty acids were used such as octanoate, palmitate and retinol, as well as different doses of TGF-β1 with concentration as high as [20 ng/ml]. Different incubation times with TGF-β1, ranging from 4, 8, 24, 48 hours, were also selected.

None of these experiments showed any evidence of loss of fat droplet in LX-2 cells upon activation by (TGF-β1) (data not shown).

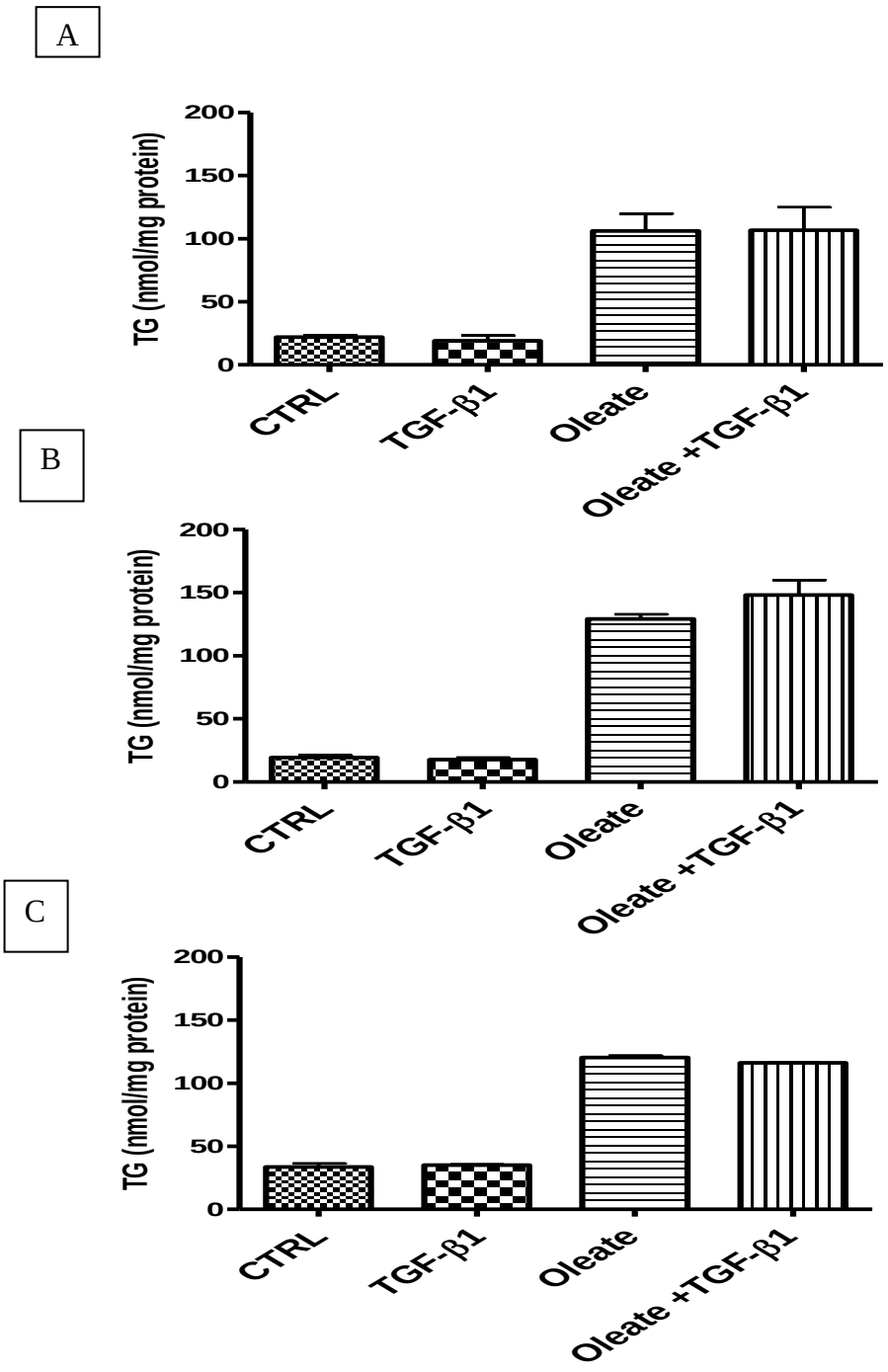

Figure A4.9: The effect of TGF-β1 on the triglyceride levels of control and fat-loaded LX-2 (A) LX-2 cells were fat-loaded with oleate [0.25mM] for 24 hours. TGF-β1 [2.5 ng/ml] were then added in the corresponding wells for another 24 hours. (B) LX-2 cells were treated with TGF- β1 [2.5 ng/ml] for 24 hours and then cells were washed and treated with oleate [0.25mM] for 24 hours. (C) In this setting, both oleate [0.25mM] and TGF- β1 [2.5 ng/ml] were added at the same time for 24 hours. Data shown are from single experiment (each data point from triplicate wells). CTRL (no-addition)

A6 (Chapter six appendix)

A6.1 Methods

General protocol: For experiments in this chapter, C3A and LX-2 cells were seeded and maintained in DMEM containing 10 % FBS as described in section 2.2.1. After 24 hr incubation (i.e. at confluence) treatments was started as described below. α-1 Pro-collagen mRNA was measured as described in section 2.2.10.

Protocol A6.1.1: LX-2 cells were incubated in IT medium for 24 hours before treatment with TGF-β1 [2.5 ng/ml]. The α-1 pro-collagen mRNA production in these cells exposed to TGF-β1 was then measured at 4, 8 and 20 hours to follow the time course of its effect.

Protocol A6.1.2: LX-2 cells were treated with various doses of t-BuOOH [0, 5, 10, 50, 100, 200 µM] for 24 hours and mRNA expression of α-1 pro-collagen, lysyl oxidase (LOX) and steroid sulphatase (STS) was assessed.

Protocol A6.1.3: C3A cells were incubated in IT medium for 24 hours prior to commencement of TGF-β1 [2.5 ng/ml] treatment in IT-medium for a further 20 hours.

A6.2 Results

A6.2.1 The temporal effect of TGF-β1 treatment on α-1 pro-collagen mRNA in LX-2 cells:

Because TGF-β1 is an important cytokine and can have a rapid effect within 4 hours on many cells, experiment was conducted according to protocol A6.1.1.

As shown in figure A6.1, the expression of α-1 pro-collagen mRNA in LX-2 cells showed a significant two-fold increase, but this required a 20 hour treatment period.

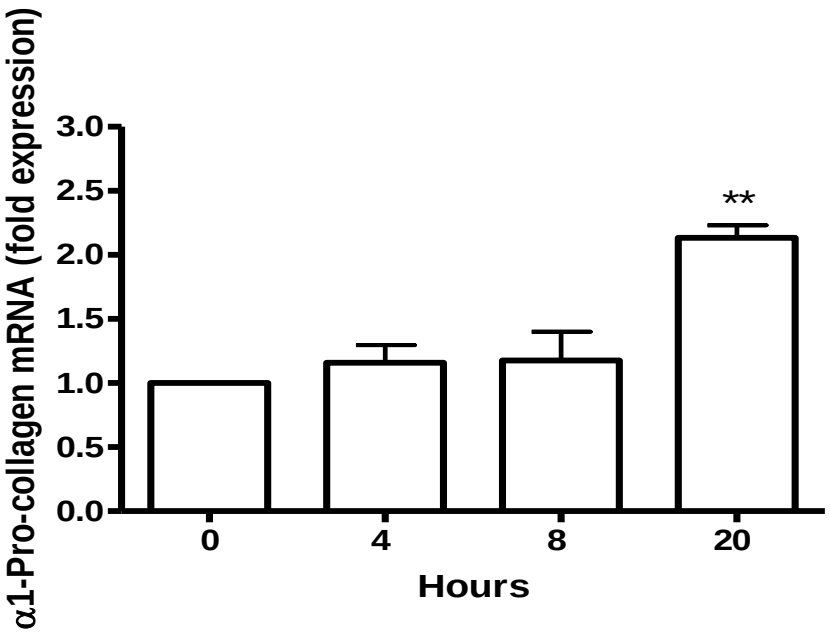

Figure A6.1: The temporal effect of TGF-β1 on the expression of α-1 pro-collagen mRNA in LX-2 cells LX-2 cells were treated with TGF-β1 [2.5 ng/ml] for 0, 4, 8 and 24 hours. The cells were then collected to measure the α-1 pro-collagen mRNA levels. Data are the mean ± standard error (± SEM) for three experiments (n=3), each experiment done using triplicate wells. ** $p<0.01$ compared to zero time (CTRL)

A6.2.2 Dose response of t-BuOOH treatment on other LX-2 gene products (lysyl oxidase, steroid suphatase)

Because t-BuOOH decreased the level of α-1 pro-collagen mRNA expression, it was essential to explore further this effect by clarifying whether this effect was specific to the expression of α-1 pro-collagen or an effect on other collagen pathway genes (e.g. lysyl oxidase), or a general effect which extended to non-collagen pathway genes (e.g. steroid sulphatase). For this purpose, an experiment was conducted according to protocol A6.1.2.

Lysyl oxidase (LOX) is a gene that expresses a copper-dependent protein that is involved in the cross-linking of collagens and elastin. Furthermore, LOX is increased in many fibrotic diseases (Smith-Mungo and Kagan, 1998).

Steroid sulfatase (STS) is responsible for the regulation of the formation of biologically active steroids. The enzyme has a pivotal role in hormone-dependent tumours including breast and prostate cancers and is found to be at increased levels in aggressive tumours and indeed is of prognostic value in these pathologies (Reed et al., 2005). STS has no link with collagen synthesis.

As shown in figure A6.2, t-BuOOH decreased the level of α-1 pro-collagen mRNA expressed by LX-2 cells in a clear dose-dependent manner. A concentration of 100 µM t-BuOOH decreased the mRNA level by about 60-70% while 200µM t-BuOOH decreased the mRNA level by 80-90%. Notably, no significant enhancement of α-1 pro-collagen expression was observed at any concentration of t-BuOOH.

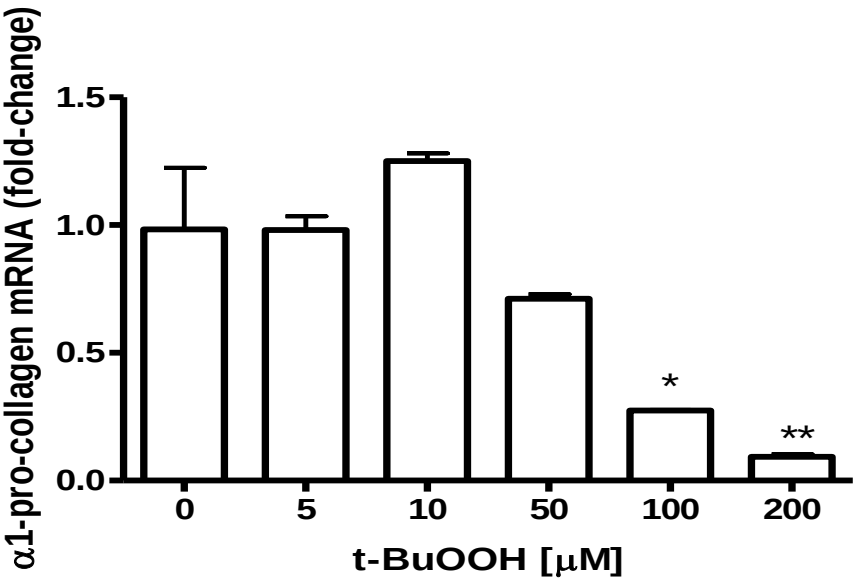

Figure A6.2: the dose-dependant effect of t-BuOOH on the expression of α-1 pro-collagen. LX-2 cells were treated with various doses of t-BuOOH [0-200 μM] for 24 hours. The cells were then collected and assessed as described in section 2.2.10. Data are the mean ± standard error (± SEM) from three experiments (n=3), each experiment done using triplicate well. * $p<0.05$, ** $p<0.01$ compared to CTRL (0 concentration of t-BuOOH).

As shown in figure A6.3, t-BuOOH significantly inhibited the expression of both STS and LOX genes. The inhibition by t-BuOOH on STS and LOX was 65%, 75% respectively compared to a 90% inhibition of α-1 pro-collagen gene expression. It was therefore concluded that t-BuOOH treatment affected the level of expression of a number of genes both within and outwith the collagen pathways.

Because t-BuOOH appeared to be a non-specific inhibitor of gene expression, it was necessary to establish whether other inducers of oxidative stress such as H_2O_2 and menadione also affected α-1 pro-collagen expression. However, neither H_2O_2 nor menadione affected α-1 pro-collagen expression in LX-2 cells (data not shown), implying that the effect was specific to lipid hydroperoxides.

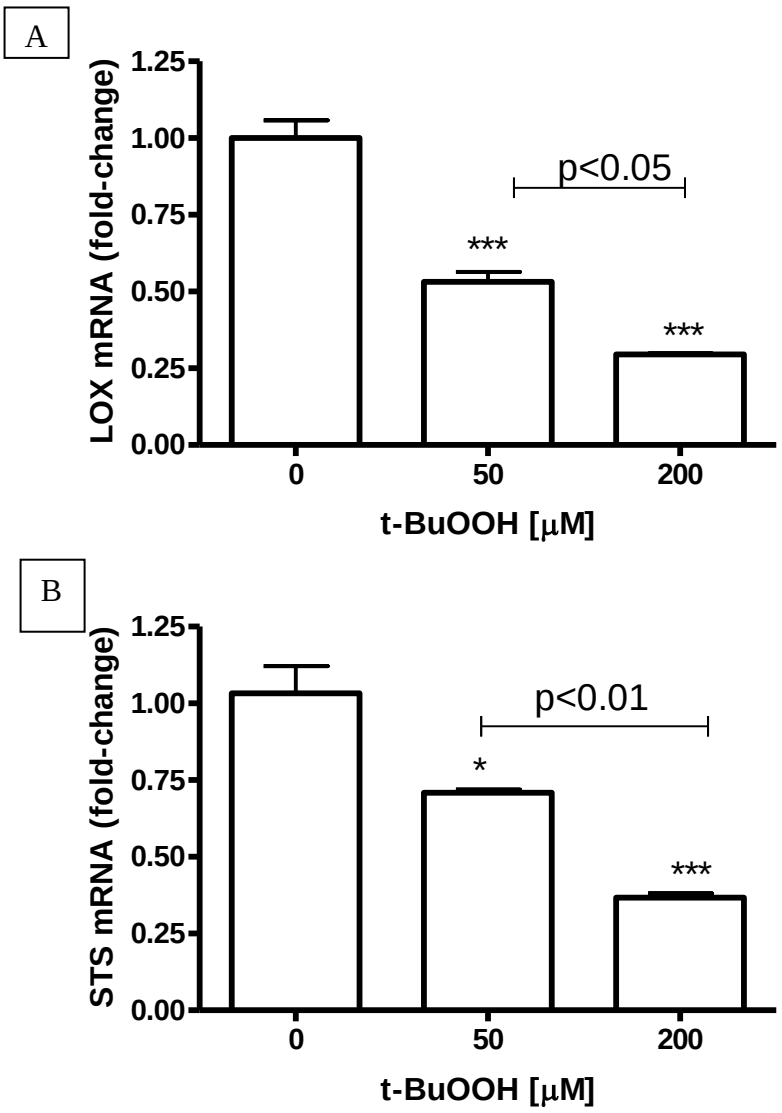

Figure A6.3: The effect of t-BuOOH on the (A) LOX and (B) STS mRNA expression LX-2 cells were exposed to t-BuOOH for 24 hours. LOX and STS gene expression was then assessed. The graph shown is a single experiment done using triplicate wells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to CTRL (0 concentration of t-BuOOH).

A6.2.3 The temporal effect of TGF-β1 on α-1 pro-collagen mRNA in C3A cells:

A pilot experiment to establish α-1 pro-collagen mRNA levels in C3A cells revealed that these cells were also responsive to TGF-β1 [2.5 ng/ml] (data not shown). A temporal study of the effect of TGF-β1 on C3A α-1 pro-collagen mRNA expression was therefore conducted. For this purpose, the experiment was conducted according to protocol A6.1.3.

As shown in figure A6.4, the expression of α-1 pro-collagen in C3A cells was significantly increased after TGF-β1 treatment reaching up to about 35 fold increase after 20 hours of treatment comparing the 20 hr exposure to time 0 hr.

The C_T value of the α-1 pro-collagen gene expression on C3A cells after 20 hours of TGF-β1 treatment was (30.28 ± 0.53). This was not significantly different from the C_T value of the untreated LX-2 cells (29.61 ± 0.53).

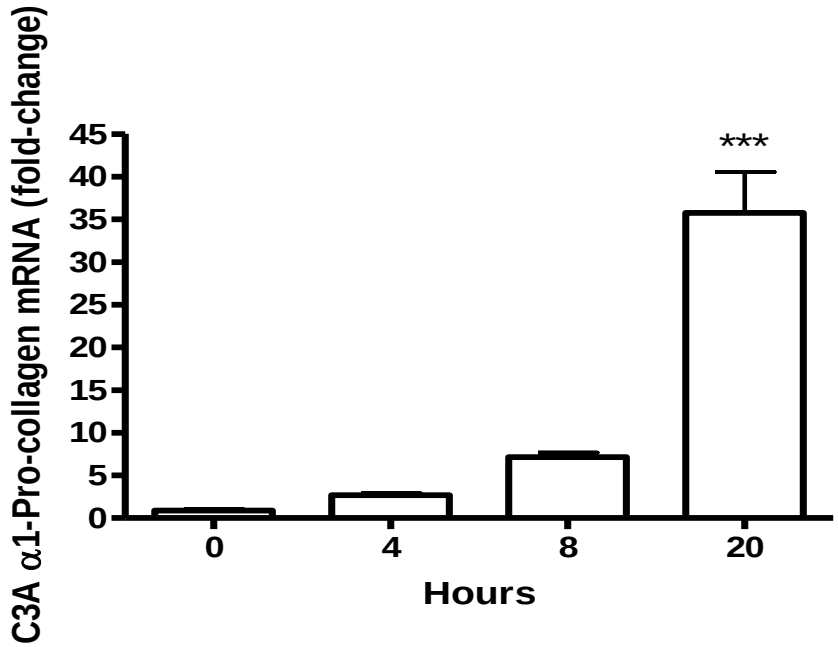

Figure A6.4: The temporal effect of TGF-β1 on the expression of α-1 pro-collagen mRNA in C3A cells C3A cells were incubated with TGF-β1 [2.5 ng/ml] for 0, 4, 8 and 20 hours. Cells were then collected to measure the level of α-1 pro-collagen mRNA. Data represent the mean ± standard error (± SEM) of three experiments, each experiment done using triplicate wells. *** $p<0.001$ compared to CTRL (0 time).